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VOLUME 27 NUMBER 3

STAIN TECHNOLOGY

A JOURNAL FOR MICROTECHNIC AND HISTOCHEMISTRY

MAY 1952

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Subjects suitable for publication in Stain Technology: microscopic technic; histochemistry; nature and uses of biological stains. Articles submitted should not have been previously published elsewhere.

Abstracts. An abstract of 100–400 words is required for every paper. Such an abstract can be utilized by abstracting services directly, and thus make sure that the author's own views are expressed. Also, it can take the place of a conventional summary at the end of the original article. When possible the abstract should give essential details of technic so that a reader can follow them in case the original article is not available. Uniformative expressions, such as "Methods for.....are listed", "The results are briefly described" have little value in abstracts. Similarly, the repetition of the title in the first line of the abstract is unnecessary.

Staining formulae should be definite and accurate, with batch or certification number of stains actually used given whenever possible — or such information as manufacturer, approximate date of purchase, if no batch number is available.

Tables. Legends should be fully explanatory so that each table is complete in itself; tables should be intelligible without consulting text.

References to literature should be carefully verified, and ordinarily the complete title and pagination should be given. Footnotes are preferred when references are few, otherwise a list of references at the end of the paper; it is appreciated if authors follow the regular style adopted by Stain Technology.

Illustrations should be numbered and the legend for each should be written on a sheet of paper loosely attached to the illustration. They should always be referred to in the text. Authors are requested to limit the size of illustrations, showing only significant, essential details; a figure containing 20–30 repetitious units may actually gain emphasis if their number is reduced to 10–20. A figure surrounded by excessive background is improved by trimming. *Colored illustrations are accepted only at the author's expense.*

STAIN TECHNOLOGY

VOLUME 27 MAY 1952 NUMBER 3

THE WORK OF THE COMMISSION LABORATORIES AT ROCHESTER, NEW YORK

H. J. CONN, *Secretary, Biological Stain Commission, Geneva, N. Y.*

Attention should be called again to the fact that although the business headquarters of the Biological Stain Commission, as well as the editorial and publication offices of Stain Technology, are still at Geneva, N. Y., its Research and Assay Laboratories were moved to Rochester in 1947, and have been there since then. They are located in the Department of Biochemistry of the University of Rochester Medical School, and are under the direction of Dr. E. H. Stotz, Head of that Department. The work is financed by means of grants made by the Stain Commission to the University of Rochester. The personnel carrying on the work consists of two full-time assistants, one graduate student supported by a fellowship, and a part-time secretary-bookkeeper.

With the exception of the work of the above-mentioned graduate student (who is engaged on special research problems of the Commission) the work of these laboratories is primarily assaying stain samples submitted to the Commission and making recommendations as to whether or not the batches represented by these samples should be certified. This involves the following steps: (1) chemical and spectrophotometric assay to learn whether the dye is true to type and of sufficient concentration for certification; (2) testing for various uses in histology, cytology, bacteriology, etc., by Miss Mary A. Darrow, who has been with the Commission for a long period and is familiar with the various types of technic involved; (3) the testing of certain stains in special procedures by skilled collaborators elsewhere; (4) finally a collation of all the information thus obtained to decide whether the sample should be approved.

Such batches as are approved in this way are officially certified by the Commission. This endorsement is indicated by the Commission's certification which appears on the labels of individual bottles of the batch in question. Any particular certification applies only to the batch for which it has been granted, not to other batches of the dye produced by the same manufacturer. While it is true that variations occur in the quality of a given stain from different companies, it is also common to find that different batches from the same company show considerable variation — often even more than

that between the average quality of the product of one company and that of any other concern. The Stain Commission rigorously maintains an impartial attitude toward the products of any particular stain manufacturer; and within its facilities, coöperates with each toward better products for scientific use.

Although Stain Technology is the official organ of the Stain Commission, neither the articles nor the advertisements therein constitute in any way an endorsement by the Commission of any particular company or batch of stain. Just as the advertisements give the advertiser's own ideas as to the merits of their products, so the views in the scientific papers are those of the authors, not those of the Stain Commission Laboratories. We have encouraged authors to be definite in giving names of manufacturers and certification numbers of batches, more to make it possible for other workers to duplicate their results than to indicate approval of any particular product.

Many papers could be cited as instances to the point; but special reference might be made to one in this issue which speaks very highly of the orcein of one particular company. There is no question in the minds of the Commission's investigators as to the high quality of this particular batch of synthetic orcein; but certification has been granted to that company only on a batch basis, and has also been granted to lots of orcein (both natural and synthetic) made by other manufacturers. It is still too early to be sure that the properties of this particular brand of that dye which are so highly commended by this author will prove constant.

It should be repeated that Commission endorsement is indicated only by its certification label. It has always been the editorial policy of this journal to exclude gross misstatements of fact from advertisements or unsupported claims about stains or methods from papers by individual authors, but to encourage authors to name the products they use, and whenever possible the actual batches employed, so as to make their methods more definite and easier for others to follow.

ORCEIN AND ELASTIC FIBERS[1]

S. I. KORNHAUSER, *Department of Anatomy, School of Medicine, University of Louisville, Louisville, Ky.*

Received for publication Sept. 3, 1951

ABSTRACT.—**Orcein was first proposed as an elastic fiber stain by Taenzer in 1890, and has proved very useful for the purpose. It is an important constituent of the author's "Quad" stain. Unfortunately not all orcein samples have proved equally satisfactory, whether they are derived from the lichens from which the dye was originally prepared or have been manufactured by a synthetic process. At the present time several brands are available, and two of the brands of the synthetic product investigated have not proved to be the same thing; one of the latter proves only fair as an elastin stain, the other is one of the best samples the author has ever tried.**

In teaching histology to medical students it has been my custom to use an elastic stain along with a general tissue stain on sections showing epithelia, muscles and other elements. This enhances the value of the slides and cuts down on the number of preparations needed in the loan collections. For the elastic tissue stain I have found orcein the most versatile, reliable and simple of the various commonly used stains for elastic fibers.

Besides orcein, resorcin-fuchsin (Weigert, 1898), resorcin-crystal-violet (Sheridan, 1929) and iodine-iron-hematoxylin (Verhoeff, 1908) are the three elastic tissue stains in general use. All four have had various modifications in the years following their introduction. Most of these changes in the original formulae are given in the standard texts on microtechnic and will not be elaborated on here. Next to orcein, the iodine-iron-hematoxylin stain for elastic tissue is undoubtedly the best. Its black color allows the elastic tissue to show up well, even when used in conjunction with the Mallory connective tissue stain or its numerous modifications. Only when one wishes to add a silver reticular stain, such as Foot (1924) or Laidlaw (1929), which give a black stain to the reticular fibers, must the iodine-iron-hematoxylin actually be avoided. Here orcein is the choice stain for its deep red color gives a fine contrast for visual work or color photography with the black reticular fibers and the blue or green color of the collagen fibers if stained by the Mallory or modified Mallory stains which generally employ anilin blue, light-green or fast-green for collagen.

My preference for orcein over the resorcin-fuchsin and resorcin-crystal-violet stains is based on several facts. First, if one makes the resorcin-fuchsin or resorcin-crystal-violet precipitate himself from which the acid alcoholic solutions are prepared it is a messy process. Secondly, the resorcin-fuchsin precipitate gives, in different batches,

[1]Aided by a grant to the University of Louisville from the Kentucky State Medical Research Commission.

solutions which vary considerably in their staining; some are dark blue, others purple, and still others almost black. The resorcin-crystal-violet gives a green stain, but this is a rather translucent green, and it is with difficulty that the finest elastic fibers are followed. It cannot be used to advantage when collagen is stained blue or green. Both of these aniline elastic tissue stains have another fault. Some uncombined basic dye always seems to remain in the precipitate after washing with water, and this stains tissue elements other than the elastic fibers. It is removed from the sections with difficulty and the decolorization may weaken the intensity of the stain in elastic fibers.

Orcein as an elastic fiber stain was developed in Unna's laboratory by Paul Taenzer and first reported at a meeting of the German Naturalists and Physicians in Bremen by Unna (1890) in September, 1890. It was an acid alcoholic solution. In 1891 and 1894 Unna (1891, 1894) published modifications of the Taenzer formula in which he increased the alcoholic content of the solution and used hydrochloric acid instead of nitric acid. The 1894 version is the one which has been generally used. It is a simple mixture; absolute ethyl alcohol – 100 ml., concentrated HCl – 1 ml., and orcein – 1 gram. Mann (1902), in his famous volume on Physiological Histology, however, preferred the original Taenzer formula.

Orcein, hematoxylin and carmine are the chief natural dyes still in general use. Orcein is made from archil extract obtained from various species of the genus *Roccella*. The archil extract has been imported by our dye companies. Orcein is closely related to litmus, is amphoteric, redder in acid solution, bluer in alkaline media. It stains from bright transparent red, in what I consider the poorer orceins, to an opaque maroon in the best products. For testing the quality of the various orceins, I have used sections of Zenker-fixed human skin in 10 μ paraffin sections. The visibility of the finest branches of the elastic fibers in the dermis approaching the basal layer of the epidermis has been my criterion of excellence. A weak, transparent stain will fail to show these well; a strong, opaque dye will reveal them nicely.

I became especially interested in orcein while developing the "Quad" stain (Kornhauser, 1943, 1945), a modified Mallory connective tissue stain with an elastic tissue stain added. Orcein contrasted well with alizarine blue 2B, fast green FCF and orange G, and other dyes used. The preparations made splendid Kodachrome transparencies which were used in teaching and presenting papers before groups. Later, while investigating the attachment of stratified epithelia to the underlying tissues and the attachment of muscle fibers to tendons and to coarse elastic fibers, we have added the staining of the reticular fibers with ammonia-silver to the "Quad" method. This demonstrates elastic, collagenous and reticular fibers, nicely contrasted in color on the same slide.

In developing the "Quad" stain, sections of a child's kidney were used, some fixed in Zenker's fluid and some in Helly's fluid. At first

orcein was used in the Unna 1894 formula, but this caused wrinkling and loss of many sections. After a number of trials I devised a mixture which avoided this trouble and which would stain deeply and selectively in one hour at room temperature. The formula is ethyl alcohol 90% – 100 ml., concentrated HNO_3 – 0.4 ml., and orcein – 0.4 grams. A vial of Grübler's orcein produced prior to World War I, which was available, was splendid; it was sparingly used. As this was getting low I tried out the American orceins. The National Aniline product, lot 2983, was the best domestic orcein, but was a brighter red and more translucent than the old Grübler sample. Importation from abroad was prevented by World War II. Soon also archil extract became unavailable to the dye manufacturers and orcein was practically unobtainable.

At this point the MacAndrews and Forbes Company of Camden, New Jersey, which had previously cooperated so nicely with the Biological Stain Commission and which had produced excellent hematoxylin and hematin (which the Commission certified) and good quality Brazilin, started work on the synthesis of orcein.

It was doubly important to get orcein because La Cour (1941) had introduced its use in strong acetic acid solution for the staining of chromosomes in smear and crush preparations of fresh plant and animal tissues with great success. Its use in place of the time-honored acetic-carmine and acetic-iron-carmine solutions became very popular. The numerous papers on this use of orcein, shortly after La Cour's and in the ten years following, indicate that the method is here to stay and is much worth while.

During 1942 a number of trial samples of synthetic orcein, produced by MacAndrews and Forbes, were sent to the author to test as an elastic fiber stain. Almost immediately it was evident that their product was going to be successful. In June of 1942 the batch FOr–1 proved to be excellent, and was certified by the Commission. It gave the same deep maroon color as the prized Grübler sample and stained very well in one hour at room temperature, using the author's formula. A second batch, FOr–2, was produced, tested and certified. There was no question about the superiority of FOr–1 and FOr–2 over the natural American orceins for elastic tissue staining. Some differences of opinion were held on their performance in the acetic-orcein stains for chromosomes. Some cytologists, however, found them very satisfactory.

With the production of fine synthetic orcein the problem seemed to be solved. But shortly after the close of World War II, MacAndrews and Forbes decided to quit the production of biological stains and sold the remaining stock of FOr–2 along with the method for its production to the Eastman Kodak Company. The limited quantity of FOr–2 was offered by at least two firms in gram lots at three dollars or more a gram. Even at this figure it soon ran out. Eastman's chemists could not make the MacAndrews and Forbes synthesis of orcein work, and track of the man who had developed

the method for MacAndrews and Forbes was lost. The Eastman people finally turned their data over to the National Aniline Company. Late in 1950 and early in 1951 I tested a number of National's trial samples. In general, most of them were about as good for elastic tissue as the natural American orceins produced before 1939, but not so deep in color or so specific for elastin as the MacAndrews and Forbes FOr–1 and FOr–2 or the old Grübler natural orcein.

Evidently the Hartman-Leddon Company also were working on the synthesis of orcein. On January 20, 1951, I tested a sample of Hartman-Leddon's synthetic orcein, number LOr–3. To my delight it proved to be superb. The firm stated that the new orcein had been produced by a new and simpler process. In March, 1951, a new batch, LOr–5, also proved to be as good as LOr–3, and it was certified by the Biological Stain Commission. These last named batches are equal as elastin stains, if not superior, to the MacAndrews and Forbes synthetic orceins and the original Grübler sample. Recently I acquired a second ten-gram vial of the Grübler pre-1914 natural orcein, which was as good as the first, both excellent performers even though thirty-five years or more old.

Once again the problem appears to be solved (at least on an empirical basis), and Dr. H. J. Conn states that some of these latest batches are fine also for chromosomes. Even though the cost is quite high, still one can make 250 ml. of solution from a gram of the dye using the author's formula, and this is sufficient for a great many sections. Let us hope that this time the process of synthesizing good orcein will not become a lost art among the manufacturers.

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STAINING AND MOUNTING HELMINTHS

DONALD D. DEMKE,[1] *Department of Veterinary Parasitology, Texas A. and M. College, College Station, Texas*

Received for publication Aug. 30, 1951

ABSTRACT.—**The following procedure is recommended: Fix cestodes and trematodes (while held flat between glass slides) 0.5–2.0 hr. in the following mixture: formalin, 15; acetic acid (gl.), 5; glycerol, 10; 95% ethyl alcohol, 24; distilled H_2O, 46; all proportions by volume. After freeing them from the slides, wash thoroughly in running water and stain immediately thereafter. Stock staining solution: ferric ammonium alum (violet cryst.), 2 g.; distilled H_2O (cold) 100 ml.; after solution, add 2 ml. concentrated H_2SO_4, bring to a boil; add 1 g. coelestin blue B (Nat. Aniline), boil 3–5 min.; cool and add 10 ml. absolute methyl alcohol and 10 ml. glycerol. Dilute 1 vol. with 3 vol. distilled H_2O for use. Stain 5–30 min., depending on size of specimens. Wash with 2 changes 0.5 hr. each of distilled H_2O, then 50% isopropyl alcohol 12–16 hr., 50% isopropyl alcohol 2 hr., followed by graded isopropyl alcohol for dehydration. Ether: ethyl alcohol (equal parts), 1 hr., is followed by embedding in celloidin in a sheet just thick enough to cover the specimens. Trim embedded specimens and dehydrate with isopropyl alcohol, 80%, 90% and absolute. Clear in beechwood creosote. Mount in balsam with cover glasses that overlap the edges of the celloidin 1–2 mm. While drying at 37°C., refill edges of mount with fresh balsam as needed. When dry, remove excess balsam and ring the edges with ordinary gloss enamel paint.**

The fragility of the resulting specimens and other difficulties with the usual methods for preparation of whole mounts of helminths, both for reference and for use in the classroom, prompted investigation of available means of obtaining suitable results. Becker and Roudabush[2] and Riser[3] have described methods of fixing, staining, and mounting which, with slight modifications, were found to be highly satisfactory for this purpose.

PROCEDURE

Cestodes and Trematodes. Fresh specimens of cestodes are placed in water in the ice-box still attached to short segments of washed intestine until hydration of mucus and partial decomposition of the

[1]Graduate Assistant.

[2]BECKER, ELERY R., and ROUDABUSH, ROBERT L. 1945. Brief Directions in Histological Technique. The Collegiate Press, Inc., Ames, Iowa.

[3]RISER, NATHAN W. 1950. Notes on toto-mount technique. Proc. Helminth. Soc. Washington, 17, 132.

mucosa make it possible to remove the scolices by gentle traction. Depending upon the specimen, this period may range from four hours *(Taenia)* to over-night *(Dipylidium)*. This procedure also relaxes the segments. Trematodes, if attached to the mucosa, are handled in the same manner, but tissue parasites are dissected out before being placed in water.

Following relaxation, cestodes are further stretched and flattened by winding them spirally around a $2 \times 3''$ glass slide, placing other slides on both sides, and applying pressure by binding the ends of all three slides with rubber bands. Three or four trematodes of approximately the same size and thickness may be flattened by binding between two $2 \times 3''$ slides. All specimens should be kept wet during this phase.

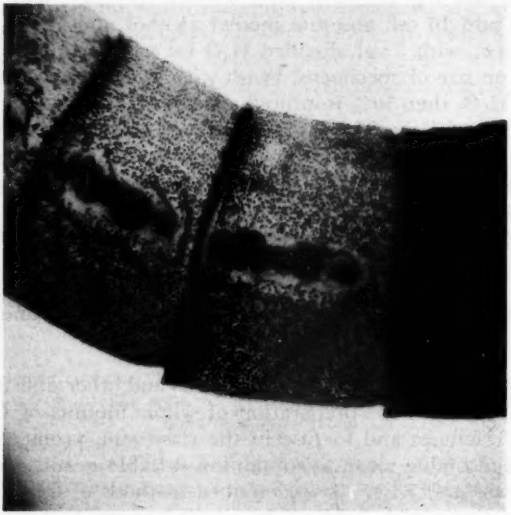

FIG. 1. — *Diphyllobothrium mansoni*, stained gravid segments.

The bound specimens are next immersed for 30 minutes to 2 hours in a jar containing the following fixative and preservative: 95% ethyl alcohol, 24 parts by volume; formalin, 15 parts; glacial acetic acid, 5 parts; glycerol, 10 parts; and distilled water, 46 parts[2]. Care should be taken, initially, to see that all air bubbles are removed from between the bound slides so that the fixative can come in contact with the specimens. This may be accomplished by inserting a razor blade or other thin, sharp object between the upper ends of the bound slides until the entrapped bubbles have risen to the surface. This also lets the fixative replace any capillary water surrounding the specimens between the slides. They are freed from the slides

and washed in running water for one-half to one hour, depending upon size, just before staining. If it is so desired, the specimens may be stored indefinitely in fresh fixative and preservative.

Staining of both cestodes and trematodes is done in Riser's modification of a solution suggested by Dr. F. Proescher.[3] This technic has been further modified by diluting the stain with distilled water. One part of stock staining solution to three parts of water seems to give the best results and tends to prevent the extreme overstaining often encountered when using the stock solution described by Riser. Stock solution is prepared as follows:

Dissolve 2 g. of ferric ammonium sulfate (violet crystals) in 100 ml. of cold distilled water. Add 2 ml. of cencentrated sulfuric acid. Bring

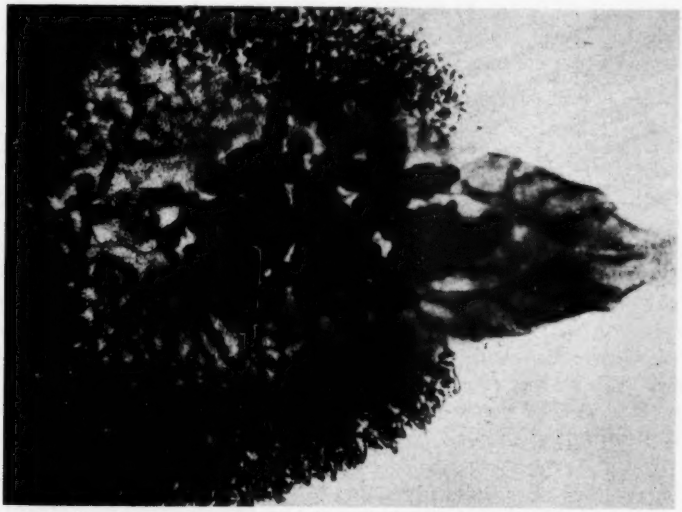

FIG. 2. — *Fasciola hepatica,* anterior end, dorsal view, stained specimen.

to a boil and add 1 g. of coelestin blue B (National Aniline Division, Allied Chemical and Dye Corp.). Boil for a few minutes. Cool and add 10 ml. of absolute methyl alcohol and 10 ml. of glycerol.

Staining is accomplished by immersion in the diluted solution from 5 to 30 minutes, depending upon the size of the specimen, e.g., scolices and immature proglottids of *Taenia hydatigena* and *Dipylidium caninum,* 5 minutes; mature and gravid proglottids of *T. hydatigena, T. saginata, D. caninum, Diphyllobothrium mansoni* (Fig. 1), and *D. latum,* 10 minutes; mature *Fasciola hepatica* (flattened specimens ½ by 1½ inches), 30 minutes (Fig. 2).

Following staining, the helminths are passed through at least two changes of distilled water, leaving them one-half hour in each change

or until no further stain is removed. They are then transferred to 50% isopropyl alcohol, two changes, remaining in the first change 12 hours (or over-night) and in the second change 2 hours. These steps remove the excess stain and give excellent differentiation.

The specimens are next passed through an ascending series of isopropyl alcohols (70, 80, 95%) into absolute isopropyl alcohol. One hour in each change seems to be sufficient. After absolute alcohol, they are placed in ether-alcohol (absolute ethyl alcohol and ether, equal parts) for an hour, and then transferred to a Petri dish or other suitable flat container. Enough thin celloidin (3%) is immediately poured on to cover them. The specimens are arranged in the celloidin so that they are widely separated; the dish is covered, and

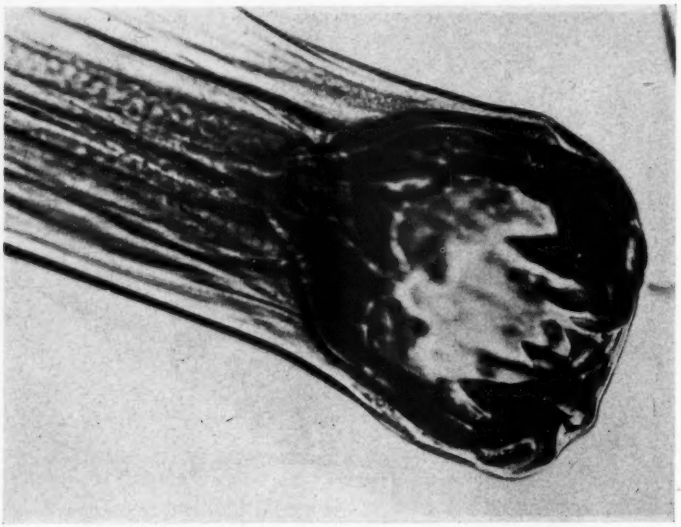

FIG. 3. — *Ancylostoma caninum,* buccal cavity, ventral view.

the ether-alcohol allowed to evaporate very slowly. In all events, the film should be sufficiently thick to completely cover the specimen and serve as a support for the cover slip. Care must be taken at this point since too rapid evaporation of the ether-alcohol causes bubble formation in the celloidin, which interferes with observation in the finished mount. After the celloidin has become firm, it is flooded with 80% isopropyl alcohol for 30 to 45 minutes.

Sections or areas of the celloidin (of appropriate size and shape) containing an entire specimen are cut out. The sections should be 1 to 2 mm. smaller on all sides than the cover glasses to be used. They may be stored, until mounting, in 80% isopropyl alcohol.

Prior to mounting, the sections are dehydrated in 90% and abso-

lute isopropyl alcohol, one hour in each, and cleared in beechwood creosote (until the sections sink). They are mounted in xylene balsam on 1 x 3 inch slides. Beechwood creosote is preferred to xylene or oil of cedarwood for clearing as it renders the sections more pliable and easier to handle.

After mounting, the slides are placed in a 37°C. incubator, examined daily, and xylene balsam added to replace the beechwood creosote and xylene that evaporates. This procedure is continued until no further evaporation occurs and the balsam has hardened. During this process the celloidin may darken appreciably; however, this does not produce any apparent damage to the specimen. After the balsam has hardened, the excess is removed by scraping and the cover glasses ringed with ordinary gloss enamel paint.

Since the celloidin sections occupy most of the area under the cover glasses, no additional support is needed for the specimens, and, should the cover be broken, it is a relatively simple matter to remove and remount the celloidin sections.

The possibility of mounting specimens in celloidin prior to staining was investigated, but it was found that the celloidin absorbed an excess of the stain which was practically impossible to remove without materially damaging the specimen.

Nematodes. Fresh specimens are killed in hot 70% isopropyl alcohol, passed through the ascending series of alcohols into ether-alcohol and celloidin, cleared and mounted in the same manner as cestodes and trematodes with excellent results. It was found that nematodes that had been stored for long periods of time in either alcohols or formalin yielded excellent preparations when processed by this method (Fig. 3). Those specimens stored in formalin were first washed one to two hours in running water and then transferred to the ascending series of isopropyl alcohols beginning with 50% alcohol.

MAPPING GOBLET CELLS IN MUCOUS MEMBRANES[1]

H. Moe, *Department of Anatomy, Faculty of Medicine, University of Copenhagen*

Received for publication Oct. 1, 1951

Abstract.—**A method is described for determining the number of goblet cells of the villi and crypts of Lieberkühn in the small intestine with an accuracy far exceeding that which appears to be possible by counting on tissue sections. In groups of intact villi or crypts, previously isolated by microdissection, the goblet cells are stained, with as little staining as possible of the other tissue elements; thereafter the preparations are made transparent by embedding in a medium possessing a refractive index similar to that of the tissue. The staining is performed by the McManus-Hotchkiss periodic-leucofuchsin method (1948) with the modification that Schiff's reagent is diluted with 3 parts of water, the staining period cut down to 2½–3 minutes, and the rinsing with bisulfite solution to 4–6 minutes. The embedding medium consists of colophonium and quinine hydrochloride in anise oil (Aurell, 1938). By this procedure, all the stained cells of the preparation may be visualized by manipulating the fine adjustment of the microscope. Counting of the goblet cells of the villi may be performed with great accuracy by projecting the picture of the preparation from the microscope on sectional paper and placing dots in the positions of the stained cells. The degree of magnification is determined by a corresponding projection of the scale of a micrometer disc.**

Introduction

Determination of the number of cells in tissues by the ordinary procedure of studying the number of cells in serial sections is subject to several sources of error. It is often difficult to correct for such errors to the extent of making the results sufficiently reliable to be applicable as a basis for physiological studies or other studies requiring exact analysis. The development of more specific staining methods for given types of cells has opened up new roads for simpler and more exact methods for determining the distribution and number of cells in tissues, since thick tissue sections or well-defined pieces of tissue (such as intestinal villi) may be stained to show special forms of cells and then made transparent to allow inspection of all the stained cells. This paper describes the application of these principles for determining the number and distribution of goblet cells in certain mucous membranes.

[1]Aided by a grant from the King Christian X's Foundation.

To stain the mucus of the goblet cells, the author used a slightly modified form of the method published by McManus (1948). In an endeavor to avoid as far as possible the staining of other tissue elements and not to compromise the possibility of making the tissue transparent subsequently, Schiff's reagent was diluted with 3 parts of water and the staining period cut down to 2½–3 minutes. The best results seemed obtainable when the staining was performed at

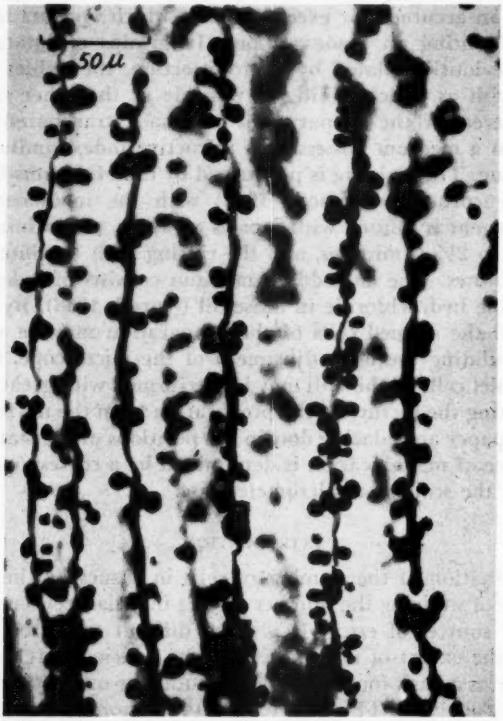

FIG. 1. — Goblet cells on the upper surface of a whole villus from the duodenum of a cat. Since the photomicrograph is made with sharp focusing on the upper surface of the glassy transparent villus, the goblet cells of the under surface can only be discerned as vague patches. Note the distinct appearance of the shape of the mucous part of the goblet cells at the lateral borders of the villus.

a low temperature. Therefore, both Schiff's reagent and the water used for dilution were stored in a refrigerator before dilution which was performed immediately before staining. Moreover, the metabisulfite rinse was reduced to 4–6 minutes. By this method an intense staining of the goblet cells was obtained without troublesome staining of other tissue elements.

Preparation of Specimens from Villi and Crypts

The specimens are obtained from the small intestine of cats. Pieces of the intestine are removed, cautiously cut open, and fixed, hanging, in alcohol-formalin solution (one part commercial formalin to two parts 95–96% alcohol) for 24 hours; then transferred through 95–96% alcohol to absolute alcohol, still hanging to avoid deformation of the villi. Small pieces of intestine are cut off and placed,

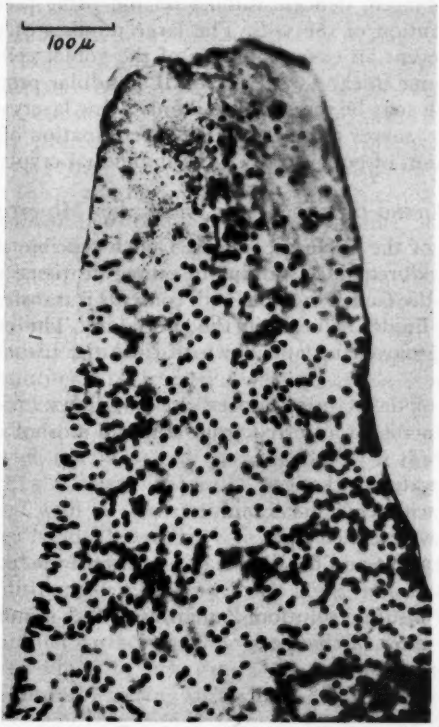

Fig. 2. – Goblet cells in intact crypts of Lieberkühn from the duodenum of a cat. Of course, the photomicrograph cannot reproduce the plastic impression obtained by manipulating the fine adjustment which makes it possible to determine to which crypts the individual cells belong.

held by a small forceps (serrefine), in a Petri dish with absolute alcohol. The dish is placed under a stereoscopic microscope, and the villi and crypts are dissected with a micro-dissection needle. The villi are freed most simply in the form of tissue pieces consisting of a single row of about 5 villi kept together by the underlying part of the mucosa, by cautiously employing the side of the needle to get

down between the rows of villi and crypts to the muscularis mucosae from which the mucosal pieces are detached. The isolated villi are inspected for epithelial lesions, and damaged villi are removed. With a fine forceps, the preparations are transferred into a Gooch crucible placed in absolute alcohol. The holes in the bottom of the Gooch crucible must be so fine that the tissue pieces do not pass through them. With some practise 20–40 villi may be isolated in the hour.

The crypts of Lieberkühn appear in the stereoscopic microscope as densely arranged, delicate tubules on the tissue pieces obtained by the preparation of the villi. The large number of glands, however, may prevent an exact counting of the goblet cells in a tissue layer of the same thickness as the villi. If glandular preparations are desired, glands may be removed until only a few layers remain. This affords an easy survey and permits the determination of the number and distribution of goblet cells in the individual crypts.

Staining of the Preparations and Mounting

Placement of the tissue pieces in a Gooch crucible simplifies the staining procedures, since staining of numerous tissue pieces is possible because the Gooch crucible with contents is transferred through the different fluids included in the procedure. The crucible must frequently be moved up and down to rinse the tissue pieces effectively.

The steps of the staining process are as follows:

(1) Gooch crucible transferred from absolute alcohol to 70% alcohol, whence it is passed to
(2) distilled water, 3 changes,
(3) periodic acid, 0.5%, for 5 minutes,
(4) distilled water, 3 changes,
(5) Schiff's reagent and distilled water 1:3 freshly prepared, chilled; 2½–3 min.,
(6) rinsed in bisulfite solution, 2 changes of 2–3 minutes each,
(7) distilled water, 2 changes,
(8) 95–96% alcohol,
(9) absolute alcohol, 2–3 changes,
(10) benzene, 2 changes.

The times used for passing the preparations through water and alcohol must be about 10 times longer than that required to stain sections. The preparations must be left in benzene for 1–2 hours to facilitate clearing.

The clearing procedure may be carried out by one of the methods described by Aurell (1938, 1941). In the present experiments, the preparations were transferred from benzene to anise oil (about 24 hours) and mounted in a mixture of colophonium, quinine hydrochloride and anise oil with N_D of about 1.557. (Preparation: 5 g. quinine hydrochloride is dissolved by slight heating in 20 g. of anise

oil to which has been added 1 g. of pulverized colophonium. After cooling to room temperature 19 g. of colophonium is added. The resin is completely dissolved within a few days. The dissolving process must not take place at a high temperature, since this will give the solution a dark color. The colophonium must be as pale as possible).

Counting of Goblet Cells[2]

Counting of the goblet cells in the crypts of Lieberkühn may be performed under the miscroscope from the bottom to the mouth of the glands (Fig. 2). Direct counting of the goblet cells on the villi is difficult because they are often present in extremely large numbers (Fig. 1). Considerable simplification and great accuracy may be obtained by projecting the picture of the preparation from the microscope to yellow sectional paper on which the goblet cells are marked. In this way, the total number and the distribution of the goblet cells on the villi may be determined, since manipulations of the fine adjustment of the microscope will in turn visualize all the cells from the upper surface, through the lateral surfaces, and to the under surface. The axis of the villus has previously been adjusted to follow one of the lines of the sectional paper and the contours of the villus have been traced. After marking all the goblet cells, the counting is performed in the square cm. sections according to the principles that blood cells are counted in a counting chamber. The projection may be carried out with a Leitz Ortholux microscope with a drawing mirror placed above the eyepiece (in a dark room!). On most villi the determination of the number of stained cells may be performed with an accuracy approaching 100%. On a few villi, on the other hand, the goblet cells of the lateral surfaces may be superimposed upon each other to such an extent that it may prove difficult to distinguish every single cell. This, however, applies only to a few per cent of the total number of cells, provided that flattened villi are mounted surface up; not edge up. The degree of magnification is determined by inserting a micrometer disc at the site of the preparation and tracing the scale on the sectional paper. The magnification may be adjusted prior to the tracing of the cells, so that (for example) 200μ in the preparation correspond to 5 cm. of the sectional paper.

A similar procedure may be used in determining the number of goblet cells in the mucosa of the air passages. The mucosal surface of the stained and transparent preparations is projected on sectional paper and the cells are marked and counted. The counting may also be done directly, using an eyepiece micrometer disc (square type).

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[2]In this connection we shall not deal with the applicability of the method in studying the functional cycle of the goblet cells.

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A MODIFIED TURCHINI TECHNIC FOR THE DIFFERENTIAL STAINING OF NUCLEIC ACIDS

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ABSTRACT—**The preparation of the 9-methyl-2,3,7-trihydroxy-6-fluorone reagent for the selective staining of both desoxyribose and ribose nucleic acids is described. With slight variations this method follows the Duckert (1937) modification of the Liebermann and Lindenbaum (1904) reaction.**

The present modification of the Turchini et al. (1944) staining procedure has been used on human autonomic ganglia fixed in Bouin's fluid, rat tissues, fixed in Bouin's, Zenker's, formol and formol-saline fixatives and mouse liver frozen and dried. The modified Turchini method has been examined primarily for its qualitative reliability by means of the following procedure. Ribonuclease treated sections were compared with adjacent sections immersed in distilled water. In succeeding steps half of these sections were stained by the modified Turchini process and the other half by Einarsson's gallocyanin chrome alum. Evidence gleaned from this and other tests indicates that 9-methyl-2,3,7-trihydroxy-6-fluorone may be used for the selective staining of desoxyribose and ribose nucleic acids.

INTRODUCTION

The current intense interest in the nucleic acids has created a need for specific stains for the quantitative and qualitative visualization of these intracellular constituents. In connection with some problems in our laboratory a need arose for a dye which would simultaneously and differentially stain pentose nucleic acid (PNA) and desoxypentose nucleic acid (DNA). The Turchini technic, as described in the literature, appeared to be adaptable to our purpose. Attempts were made, therefore, to utilize the staining procedure as outlined by Turchini and his co-workers (1944). The 9-methyl-2,3,7-trihydroxy-6-fluorone was not procurable from commercial sources in this country and Canada and, consequently, had to be prepared before it could be tested.

PREPARATION

The preparation of the 9-methyl-2,3,7-trihydroxy-6-fluorone finally adopted by us follows closely the Duckert (1937) modification of the Liebermann and Lindenbaum (1904) reaction. The reagents are mixed in the following molecular proportion: (a) 1,2,4-triacetyl-trioxy-benzene 1 mole; (b) paraldehyde 1.25 mole; (c) ethanol, five times the combined weight of the above reagents and containing 5–10% sulfuric acid. Because of the instability of the desired fluorone, speed is sacrificed to enhance quantity of yield. Instead of heating on a water bath at 50–60°C. for one hour (Duckert, 1937), which might

diminish the yield of the desired fluorone, the reactive mixture is allowed to stand for 18–24 hours at room temperature. Thirty volumes of water are added. Because of the very slight solubility of the dye in water, it has been found that the greater the quantity of water added the more desirable are the results, due to dilution of the alcoholic solution of the fluorone. The reddish-orange dye is allowed to precipitate over a 24-hour period. It is then filtered, removing much of the sulfate of the dye along with unchanged starting reagents and other side products. A Büchner funnel facilitates this step by shortening the filtration time. The filtrate is discarded and the precipitate is dried in an oven at 37–40°C.

After drying, the precipitated dye is redissolved in the minimum volume of ethanol and filtered, thereby removing the alcohol insoluble reaction products. Thirty to 60 volumes of water are added to

TABLE 1. DATA ON PREPARATION OF THE 9-METHYL-2,6,7-TRIHYDROXY-3-FLUORONE.

Sample	Ave.* wt.	Ave.† yield	Decomposition temp.	Relative activity
	g.	%	°C	%
Reaction mixture after first filtration	11.26	—	—	0–2
First purification	6.66	63.4	290	10
Final product	6.25	59.5	319‡	100

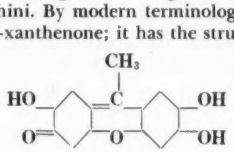
*Average of 4 preparations.

†Average of 4 preparations based on the assumption that 2 molecules of hydroxyhydroquinone triacetate yield one molecule of the 9-methyl-2,6,7-trihydroxy-3-fluorone.

‡Duckert (1937) reports that the fluorone carbonizes at 320°C.

the filtrate. The mixture is allowed to stand for 18–24 hours after which it is again filtered and the dye is dried as before. Further purification by redissolving the fluorone in a minimum volume of ethanol, filtering, adding 30–60 volumes of water and filtering again, resulted in a small loss of dye (Table 1.). The final product, after the third filtration, has the properties of the dye described by Duckert (1937). It is a reddish-orange powder which carbonizes at 319°C. It is soluble in alcohol and practically insoluble in water. Satisfactory staining with the final product was accomplished with only 10% of the amount of the first purification product required. It is therefore deemed advisable to perform two purifications when preparing the dye. This final product was employed in the experiments carried out with the dye in this laboratory.[1]

[1]We greatly appreciate the helpful suggestions of Dr. H. B. Donahoe of the Chemistry Department of St. Louis University, relative to the preparation and carbonization temperature readings of the reagent, which is here denoted by the terminology used by Turchini. By modern terminology it would better be called 9-methyl-2,3,7-trihydroxy-6-xanthenone; it has the structure:



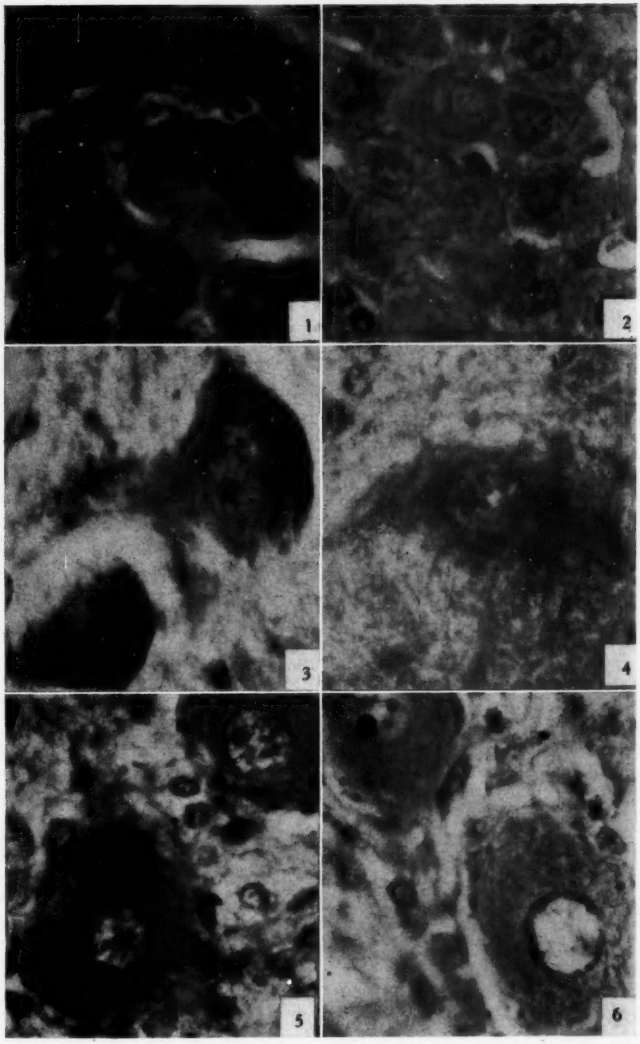


Plate I — Fig. 1–6. Turchini Stain.

1. Liver, rat, Zenker-fixed, distilled water 1 hr.
2. Liver, rat, Zenker-fixed, ribonuclease 1 hr.
3. Anterior horn cells, rat, Zenker-fixed, distilled water 1 hr.
4. Anterior horn cells, rat, Zenker-fixed, ribonuclease 1 hr.
5. Autonomic ganglion, human, Bouin-fixed, distilled water 1 hr.
6. Autonomic ganglion, human, Bouin-fixed, ribonuclease 1 hr.

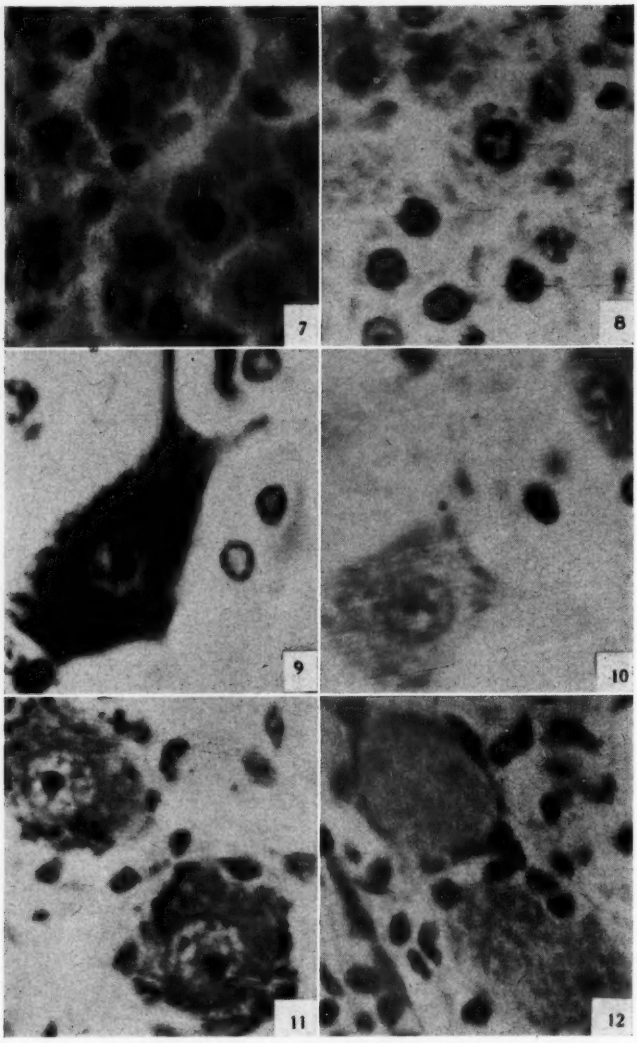


Plate II — Fig. 7–12. Einarsson Stain.

7. Liver, rat, Zenker-fixed, distilled water 1 hr.
8. Liver, rat, Zenker-fixed, ribonuclease 1 hr.
9. Anterior horn cells, rat, Zenker-fixed, distilled water 1 hr.
10. Anterior horn cells, rat, Zenker-fixed, ribonuclease 1 hr.
11. Autonomic ganglion, human, Bouin-fixed, distilled water 1 hr.
12. Autonomic ganglion, human, Bouin-fixed, ribonuclease 1 hr.

Staining Procedure

Consistent results have been obtained using the following modification of the method of Turchini et al.:

1. Paraffin sections are deparaffinized and hydrated in the usual manner.
2. Slides are immersed in 1 *N* HCl for 2 minutes at 60°C. for partial nucleic acid hydrolysis.
3. They are placed immediately in 80% ethanol for 15 seconds.
4. Slides are transferred immediately to the following solution for 20 minutes:

9-methyl-2,3,7-trihydroxy-6-fluorone,	1.0	g.
sulfuric acid	1	ml.
95% ethanol	100	ml.

   This solution, once mixed, will give satisfactory results for several weeks.
5. A 1% solution of Na_2CO_3 in distilled water for 2 minutes. A stock solution may be made up, but a portion used once for staining must be discarded.
6. The excess dye and Na_2CO_3 are removed by immersing slides in distilled water for 2 minutes (fresh each time).
7. Acetone and distilled water (50–50) for 3 minutes. (The solubility of the dye in alcohol results in extraction if ethanol is used for dehydration.)
8. Acetone for 3 minutes.
9. Acetone and xylene (50–50) for 2 minutes.
10. Two changes of xylene, 1.5 minutes each.
11. Add piccolyte and cover sections with cover glass. Sites of PNA stain a yellow to red and DNA is stained a violet to blue-black.

The use of higher concentrations of the fluorone or immersion up to 4 hours in the dye solution did not alter appreciably the localization as measured by visual appraisal of adjacent tissue sections. Deposition of the dye was, however, more pronounced after 4 hours in the fluorone solution. A fluorone concentration of less than 8 mg. (we recommend 10 mg.) per ml. of staining solution is unsatisfactory.

Results

Duplicable results have been obtained with rat tissues fixed in Zenker's, Bouin's, formol and formol-saline, human tissues fixed in Bouin's fluid and frozen-dried mouse liver.[2] Experimental rat spinal cord, autonomic ganglia and liver tissues (Alexander and Backlar, 1951) and human autonomic ganglia were stained with the above procedure. Adjacent sections were stained with Einarsson's gallocyanin-chrome-alum, hematoxylin and eosin and the Feulgen reaction. The same PNA changes observed in the above stains on experimental tissues were observed in the modified Turchini stained sections. Quantitative determination of DNA was not attempted

[2]We wish to thank Rev. Dr. L. B. Walsh, of the Department of Pathology, St. Louis University, for supplying us with the frozen-dried mouse liver.

because the presence of nuclear PNA might obscure reasonably accurate determinations. Qualitatively, the DNA showed the same localization as in comparable Feulgen stained sections.

Digestion of rat Zenker-fixed, mouse frozen-dried and human Bouin-fixed sections with ribonuclease (Worthington) permitted a qualitative examination of the Turchini stain for PNA. Rat spinal cord, liver and kidney, mouse liver and human autonomic ganglia were placed in ribonuclease, 1 mg. per ml. of distilled water (pH 6.8) for one hour at 60°C. Control sections were placed in distilled water under the same conditions for the same length of time. Both enzyme-digested and control sections from all of the above tissues were stained in Einarsson's gallocyanin-chrome alum and Turchini in succeeding steps. The visible reduction in cytoplasmic, nuclear and nucleolar stain in the enzyme-treated sections as compared with the controls for both the Turchini and the Einarsson-stained sections indicated that both of these stains owe a large portion of their staining capacity to the PNA present in the tissues, (Fig. 1–12). Residual cytoplasmic stain may be attributed to the following causes: 1. Failure of ribonuclease digestion to hydrolyze and to extract all the PNA from the tissues (Laskowski, 1951). 2. Lack of absolute specificity of the stains used and the consequent staining of other intracellular substances besides nucleic acids.

The modified Turchini staining procedure as outlined above will, in our opinion, yield duplicable results for the simultaneous staining of both groups of nucleic acids. As used in our laboratory it has produced a color differentiation between both groups of nucleic acids. Some attempts have been made to determine its reliability, by comparing Turchini-stained sections with comparable sections processed in other staining procedures and noting the same PNA differences between experimental and control sections. Ribonuclease digestion produced a partial loss of visible PNA color in Turchini-stained sections. The Turchini stain has been found excellent for routine cytological examinations. Until it is widely used and accepted, however, its value in staining PNA and DNA is open to question.

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STUDIES ON THE PREPARATION AND RECOLORATION OF FUCHSIN SULFUROUS ACID

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ABSTRACT.—**Some of the factors affecting the recoloration of Schiff's Reagent (fuchsin sulfurous acid or FSA) by formaldehyde have been studied spectrophotometrically to determine the optimal conditions for the reaction of this reagent with aldehydes.**

Of the various reducing agents utilized in the preparation of the leuco dye from basic fuchsin, sodium sulfite and bisulfite proved to be the most satisfactory for obtaining in the reagent maximal sensitivity to recoloration with minimal quantitative variation of results.

The relative proportions of reducing agent and basic fuchsin present in the leuco dye determine its sensitivity to recoloration. Under the conditions of the present experiments, greatest reagent recoloration was obtained when the leuco dye contained 0.01 mole of sodium bisulfite and 0.001 mole of basic fuchsin per 100 ml., a ratio of 10/1.

The recoloration of a given amount of FSA is related to the amount of aldehyde and the temperature of the reaction.

The present experiments indicate the desirability of standardizing the composition of FSA and the conditions under which it is used, if the results of different investigators are to be readily reproduced or compared.

The Schiff reagent is generally prepared by the action of SO_2 on basic fuchsin, a mixture of several closely related phenyl methane dyes, which results in the reduction of the quinoid ring and the formation of the colorless N-sulfinic acid of leucofuchsin sulfonic acid (fuchsin sulfurous acid or FSA). Upon the addition of aldehyde, the Schiff reagent is recolored through the formation of a new phenyl methane dye which differs from those present in basic fuchsin in both color and structure (Oster, 1947). The recoloration of FSA has been used extensively in qualitative analytical chemistry for the identification of aldehydes and has provided the basis for several technics for the histochemical localization of certain tissue constituents such as plasmals (Feulgen and Voit, 1924), desoxyribonucleic acid (Feulgen and Rossenbeck, 1924) and polysaccharides (Bauer, 1933). Although many studies have been made concerning the chemical specificity of the reaction and considerable interest has been directed to the question of the validity of the histochemical technics cited above,

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relatively little attention has been given to the various physical and chemical factors which might influence the recoloration of the leuco dye itself. Consequently there is little or no uniformity either in the preparation of Schiff's reagent or in the manner in which it is used.

The present experiments were designed to study spectrophotometrically some of the factors affecting the recoloration of FSA by formaldehyde to ascertain the optimal conditions for the use of this reagent in the analytical identification and histochemical demonstration of aldehydes.

Materials and Methods

The basic fuchsin used in these studies was obtained from the Allied Chemical and Dye Corporation and was certified for histological use by the Biological Stain Commission (NF–45). The actual dye content of the sample was 91%.

Using the methods adopted by the Commission (Conn, 1946), the dye sample was found to be an acetate salt having a maximum light

Table 1. Composition of 100 ml. of Schiff's Reagent Prepared by Different Procedures

Reference	Basic fuchsin grams	Reducing agent grams	Conc. HCl milliliters
Bauer, 1933	0.83	$NaHSO_3$, 1.66	1.39
Coleman, 1938	0.48	$K_2S_2O_5$, 0.95	0.39
Hotchkiss, 1948	0.49	$K_2S_2O_5$, 0.98	2.18
McManus, 1948	0.46	$NaHSO_3$, 0.46	0.75
Rosin, 1937	0.10	Na_2SO_3, 1.00	1.00
Shreiner and Fuson, 1940	0.05	SO_2, ± 5*	0.00

*One-half saturation at room temperature.

absorption at 538 mμ as measured with a Beckman spectrophotometer. The relative amounts of rosanilin and pararosanilin in the sample were not determined since the absorption ratios of these compounds, as determined by the electrospectrophotometer, were not available. Pararosanilin derivatives are generally the chief constituents of commercial basic fuchsin. Therefore, for purposes of subsequent calculations, it was assumed that the sample consisted of 91% pararosanilin acetate having a molecular weight of 347.4. If the dye were pararosanilin chloride (mol. wt. 323.8), the homolog commonly present in basic fuchsin which differs most from the assumed molecular weight, the maximum error in calculations due to incorrect molecular weight would be 6.8%.

The great variation in the composition of the Schiff's reagent prepared by several different methods is shown in Table 1. Because of this variation, for the purposes of the present experiments a "standard" reagent was arbitarily selected. One hundred milliliters of this

standard reagent contained 0.001 mole of basic fuchsin, 0.01 mole of $NaHSO_3$ and 0.02 mole of HCl and were prepared in the following manner. Basic fuchsin, 0.39 g. of actual dye (0.42 g. of the commercial dye containing 91% basic fuchsin), was dissolved in 100 ml. of boiling distilled water and cooled to room temperature. Any loss of water due to evaporation was restored and the solution filtered. Ninety milliliters, 0.001 mole of dye, were placed in a glass stoppered flask and 1.04 g. (0.01 mole) of $NaHSO_3$ added. After the bisulfite was completely dissolved, 10 ml. of 2*N* HCl were added and the flask tightly stoppered and placed in the dark for 24 hours. In order not to disturb the composition of the reagent, the residual color was not removed with an adsorbing agent. To prevent the loss of SO_2, the stock bottles and reaction tubes were covered at all times.

After complete recoloration of the standard Schiff's reagent with an excess of formalin, the maximum absorption of light occurred at 574 mμ as determined with the Beckman spectrophotometer. Preliminary experiments were performed at this wave-length[2] to establish a procedure for the partial recoloration of the leuco dye which could be used as a standard for measuring the effects of various physical and chemcial factors on the reaction. From these studies the following procedure was adopted: 1 ml. of standard FSA, 1 ml. of 0.02% formalin freshly prepared from stock formalin containing 36 to 38% formaldehyde, and 8 ml. of distilled water were mixed in colorimeter tubes. The intensity of the color developed was measured as the percent light absorption when compared with a blank consisting of 1 ml. of FSA in 9 ml. of distilled water. At room temperature (25 ± 1°C.) the maximum absorption was reached in 20 minutes and remained constant for at least an hour and one-half. In a series of 24 determinations the percentage of light absorption averaged 51.3 with a standard deviation of 2.3.

All determinations in the preliminary and in subsequent experiments were made in duplicate. Modifications in the preparation of the reagents or in the recoloration procedure are given in detail in the following section on observations and discussion.

Observations and Discussion

(1) Sensitivity of Schiff's Reagents Prepared with Different Reducing Agents

Various sulfur-containing compounds have been used to reduce basic fuchsin to the leuco form. In addition to the commonly advocated $NaHSO_3$ or $K_2S_2O_5$, Na_2SO_3 (Rosin, 1937), $Na_2S_2O_4$ (Wer-

[2]In the actual investigations, the quantitative determinations were made with an instrument which gave a reading of 545 at this point. This was probably due to an error in the setting of the instrument; but this cannot be checked as the spectrophotometer is no longer available. It is not regarded as a matter of any importance, as the determinations made were not affected by the error.

theim, 1922 and Alexander, et al., 1950), $Na_2S_2O_3$ (Lillie, 1948) and SO_2 (Shreiner and Fuson, 1940) have also been suggested. The reactivity of FSA prepared with these different compounds has not been investigated.

The present experiments compared under controlled conditions the degree of recoloration of FSA prepared with different reducing agents. A series of Schiff's reagents was prepared with molar equivalents of $Na_2S_2O_3 \cdot 5H_2O$ (2.10 g.), $Na_2S_2O_4$ (2.85 g.), Na_2SO_3 (1.26 g.) or $K_2S_2O_5$ (2.22 g.) substituted for the $NaHSO_3$ (1.04 g.) of the standard reagent. The amount of recoloration of 1 ml. of each variant by 1 ml. of 0.02% formalin was compared photometrically with the standard Schiff's reagent by the procedure outlined above. The results are summarized in Table 2.

TABLE 2. RECOLORATION OF LEUCOFUCHSIN PREPARED WITH MOLAR EQUIVALENTS OF DIFFERENT REDUCING AGENTS

Reducing agent	Absorption, % (574 μ)	Range	Number of determinations
$Na_2S_2O_3$	64.1	58.5–70.6	8
$NaHSO_3$ (standard reagent)	51.3	49.8–56.0	24
$Na_2S_2O_4$	46.5	40.0–58.5	8
Na_2SO_3	45.6	45.0–46.0	3
$K_2S_2O_5$	30.2	30.0–30.4	3

Recoloration by formalin of the reagents prepared with Na_2SO_3 and $Na_2S_2O_4$ did not differ markedly from that of the standard FSA. The reagent in which $K_2S_2O_5$ was used developed less color than the standard, whereas that prepared with $Na_2S_2O_3$ developed more.

Although the FSA prepared with $Na_2S_2O_3$ exhibited greater recoloration than did the other variants, it had two disadvantages as did the reagent prepared with $Na_2S_2O_4$. In each, elemental sulfur precipitated during the initial reduction of the basic fuchsin and had to be removed by filtration, interfering with control of the composition of these reagents. Furthermore, the amount of recoloration under controlled conditions varied to a greater degree than in the case of the variants prepared with other reducing agents.

It should be borne in mind that these data are based on the recoloration of Schiff's reagents which were prepared with molar equivalents of the various reducing agents studied. It is possible that optimal amounts of the reducing agents were not used. Also under other conditions of recoloration the quantitative effects of the various reagents might differ from those observed in the present experiments.

(2) EFFECT OF AMOUNT OF BISULFITE ON RECOLORATION OF FSA

It is well known that the sensitivity of the Schiff's reagent is related to its SO_2 content (Finholt and Asmund, 1943; Ely and Ross, 1949).

The optimal ratio of the amount of reducing agent to dye, however, has not been determined.

A series of modified Schiff's reagents was prepared from basic fuchsin in the usual manner except the quantity of $NaHSO_3$ was varied so as to give molar ratios ranging from 5/1 to 100/1 with respect to the dye content. The recoloration of each variant was determined photometrically by the standard procedure described above. The results are recorded in Figure 1.

The recoloration of the FSA was greatest at a ratio of bisulfite to dye of 10 to 1. Variations from this ratio had a pronounced effect on the recolorability of the leuco dye. The mechanism responsible for the increasing responsiveness of the reagent as the bisulfite content is increased up to the molar ratio of 10/1 is not known. However, the

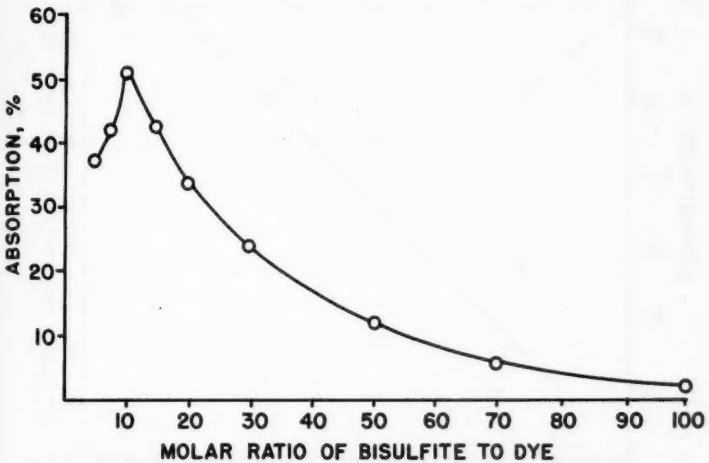

FIG. 1. Effect of bisulfite content on recoloration of fuchsin sulfurous acid by formalin.

progressive decrease in the recoloration of the reagent as the bisulfite content is increased beyond the optimum may be due to the removal of formaldehyde in the form of nonreactive bisulfite additive compounds.

(3) EFFECT OF TEMPERATURE ON RECOLORATION OF FSA

The following experiment was designed to examine the effect of temperature on the recoloration of Schiff's reagent. One ml. of standard FSA, 1 ml. of 0.02% formalin and 8 ml. of distilled water were added to each of a series of colorimetric tubes at room temperature (25.5°C.). The tubes were placed immediately in water baths ranging from 5 to 39°C. Color was allowed to develop for 20 minutes and

the tubes were then removed from the water baths and dried. The light absorption was measured immediately. The results are plotted in Fig. 2.

It is evident from the curve that the amount of FSA recolored increased with temperature. Within the range studied, the relationship was linear. These observations are contrary to those of Ely and Ross (1949) who reported that an inverse relationship existed between temperature and the amount of FSA recolored. However, these authors used desoxyribonucleic acid hydrolysate as their source of aldehyde so their experimental conditions are not strictly comparable to those of the present experiments.

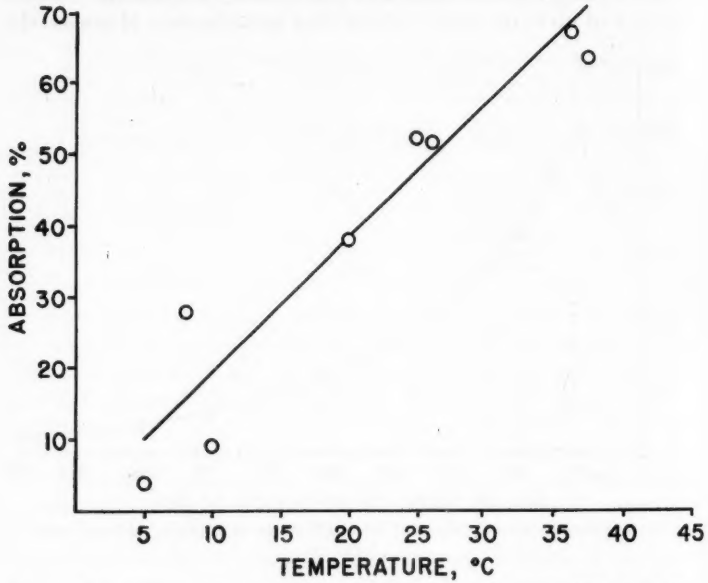

FIG. 2. Effect of temperature on recoloration of fuchsin sulfurous acid by formalin.

(4) RELATION OF AMOUNT OF FORMALIN TO RECOLORATION OF FSA

Relatively little systematic work has been reported on the quantitative recoloration of FSA by aldehydes. It is known that a wide variation exists in the minimal quantities of various aldehydes detectable by spot testing with Schiff's reagent (Feigl and Zappert cited in Feigl, 1939; p. 280). That the differences in amounts detectable are not due to differences in molecular weight alone is indicated by the fact that the minimal amounts of ortho-, meta- and para-hydroxybenzaldehyde detectable by this method are 100, 50 and 1000, respectively. In a study of the recoloration of FSA by aldoses, Rumpf (1935)

found that the intensity of color produced is not proportional to the amount of aldehyde used. Finholt and Asmund (1943), on the other hand, report a variation in recoloration of only 10% when FSA is acted upon by formaldehyde.

In the present experiment, 1 ml. of standard FSA was recolored with from 0.25 to 2.0 ml. of 0.02% formalin. The total reaction volume was maintained at 10 ml. by addition of distilled water. The results are plotted in Fig. 3. It can be readily seen that within the ranges studied there is a direct linear relationship between the amount of formalin used and the amount of FSA recolored.

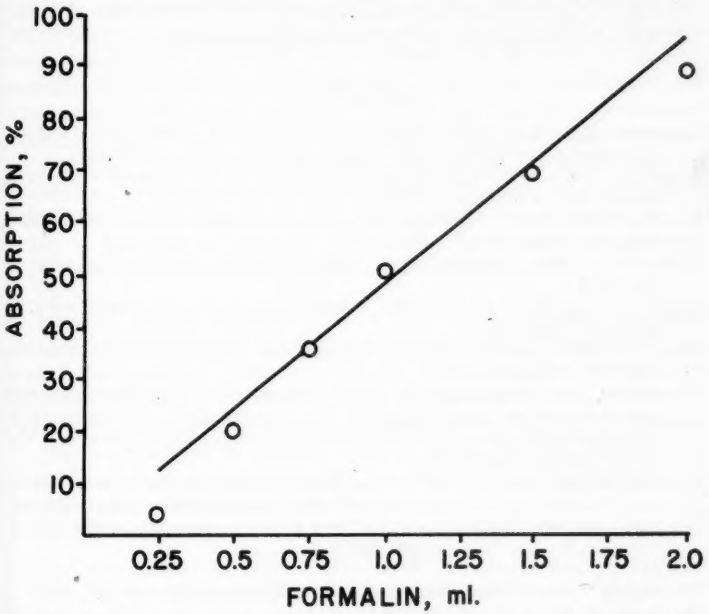

FIG. 3. Effect of amount of formalin on recoloration of fuchsin sulfurous acid.

GENERAL COMMENTS

It is evident from the present experiments that the results obtained with FSA either as an analytical or histochemical reagent may be greatly influenced by both the preparation of the leuco dye and the conditions under which it is used. In the identification of aldehydes by the Schiff's reagent spot test, as in the case of all such methods, the sensitivity of the reaction is of prime importance. The minimal amounts of various aldehydes detectable by this technic might be reduced if the test were applied under optimal conditions depending

upon the concentrations of reducing agent and aldehyde, temperature, etc.

The use of FSA as a histochemical reagent raises several problems. Different aldehydes are known to react differently with FSA. It may be possible to improve the nucleal, plasmal and other similar technics by the development of Schiff reagents better suited to the specific aldehyde to be stained. Another problem is that of the histological use of the aldehyde-FSA reaction quantitatively. This goal cannot be fully attained unless more rigid control of technical procedures is maintained. Whether quantitative determinations are a primary objective or not, technical improvements are desirable if the results of work by a particular laboratory are to be reproducible or those of different laboratories are to be readily compared.

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EFFECTIVENESS OF SCHIFF VARIANTS IN THE PERIODIC-SCHIFF AND FEULGEN NUCLEAL TECHNICS

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ABSTRACT.—**The presence of additional SO_2 in Schiff reagents above that required for bleaching of the basic fuchsin has no significant effect on the behavior of such reagents in performing the periodic-Schiff or Feulgen nucleal reactions. Schiff reagents made from aqueous solutions containing less than 0.125% basic fuchsin will not demonstrate in these reactions all the tissue elements stained by stronger solutions. Schiff reagents made from aqueous solutions containing more than 0.5% basic fuchsin will in time precipitate considerable amounts of leucofuchsin. Fresh activated charcoal discriminates much more sharply between the yellow contaminant which occurs in basic fuchsin when the fuchsin has been converted to the leuco state than it does when the fuchsin is in the colored condition. Precipitation of leucofuchsin occurs at pH 3 or slightly above in Schiff reagents of generally useful concentration, but at lower levels down to the pH of 10 *N* HCl, hydrogen ion concentration does not influence their histochemical effectiveness.**

The recognition by Lillie (1951a) of the blocking effect of sulfite ion on the aldehydes of the Feulgen and periodic-Schiff reactions has raised the question whether or not the excess of sulfite contained in all Schiff reagents (see Table 1) is not detrimental to their sensitivity. This paper reports an investigation of this point, and the significance in the same respect of fuchsin concentration, effect of decolorizing procedures, and variation of pH, concerning which problems arose in the course of the original study.

MATERIALS AND METHODS

The Schiff reagents used in this study were, unless otherwise specified, variants of the "standard cold Schiff" (Lillie, 1951b) routinely used in this laboratory. All of the experiments herein were done with basic fuchsin (C.I. 677) from certified batch NF 57.

The behavior of the Schiff modifications was examined after Feulgen hydrolysis in sections of guinea pig or dog eyes prepared with non-chromic fixatives. These contain in the nuclei of the corneal epithelium, retinal layers, and ciliary processes a series of objects which react with graded intensities. The behavior of the variants in relation to the periodic-Schiff reaction was tested in sec-

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tions of the trachea and thyroid of either cat or guinea pig, which display a variety of periodic-Schiff reactive materials in their reticulin, muscle glycogen, cartilage, mucin, collagen, parakeratohyalin and thyroid colloid.

The following were routine treatments for slides in the performance of these technics: In the *Feulgen:* (1) dewax, hydrate; (2) 1 *N* HCl (preheated) at 60°C., 20 minutes; (3) Schiff reagent, 10 minutes; (4) 0.5% $Na_2S_2O_5$, 2 minutes, 30 seconds, 30 seconds; (5) running tap water 15 minutes; (6) dehydrate, clear and mount in polystyrene. In the *periodic-Schiff:* (1) dewax, hydrate; (2) 0.03 *M* HIO_4, 10 minutes; (3) wash in water 5 minutes; (4) Schiff reagent, 10 minutes; (5) 0.5% $Na_2S_2O_5$, 2 minutes, 30 seconds, 30 seconds; (6) running tap water, 15 minutes; (7) dehydrate, clear and mount in polystyrene.

TABLE 1. COMPARISON OF VARIOUS SCHIFF REAGENTS (BASED ON 100 ML. OF REAGENT)

Author	mg. Fuchsin	mg. Bleach	Sulfite: Fuchsin (moles)	mg. Charcoal	Charcoal: Fuchsin (grams)	Bleach	N/1 HCl	pH
Alexander et al (1950)	500	500	3.53	250	0.5	$Na_2S_2O_4$	0	4.80
Barger & DeLamater (1948)	250	414	5.16	500	2.0	$SOCl_2$	0	1.24
Coleman (1938)	476	952	6.68	238	0.5	$K_2S_2O_5$	4.8	—
Margolena (1932)	455	455	3.57	0	—	$NaHSO_3$	4.6	—
Scott (1945)	100	1000	29.4	(Fuller's Earth) 100	1.0	Na_2SO_3	11.6	2.0
Stowell (1945)	455	910	6.68	227	0.5	$K_2S_2O_5$	9.1	—
De Tomasi (1936)	455	455	3.34	0	—	$K_2S_2O_5$	9.1	—
Wertheim (1922)	1.7	2000	4.50	0	—	$Na_2S_2O_4$	0	—
New Gould Medical Dictionary (1950)	25	c.600	c.140	0	—	SO_2	0	1.45
Lillie (1951b)	1000	1900	7.41	500	0.5	$Na_2S_2O_5$	15.0	2.3

EXPERIMENTAL

1. SULFITE CONCENTRATION

Low Concentration: Weiland and Scheuing (1921) and Finholt and Thorvik (1943) have both reported that without at least very small excesses of sulfite, Schiff reagents *in vitro* are inactive toward aldehydes. To assess the effectiveness of "low sufite" Schiff reagents for histochemical use it was found necessary to establish the lowest sulfite concentration at which all the fuchsin in solution could be bleached. Because of the length of time required for bleaching at

minimum sulfite levels (about 2½ days) precise determination of equivalence was difficult. It was possible to establish however the moles of SO_2 required to bleach 1 mole of basic fuchsin (considered as pararosanilin acetate) as between 1.1 and 1.4.

Schiff reagent prepared with this level of sulfite was found to be much reduced in activity with respect to strong formaldehyde. Employed in the periodic-Schiff and Feulgen technics, however, there was little change in the staining power of the reagent. Schiff reagents prepared with normal quantities of sulfite and then deprived of all excess SO_2 by the addition of iodine until a permanent yellow coloration was obtained, showed reduced intensity of reaction in tissue sections, but no disturbance of the characteristic localizations occurred.

The effect of diffusion loss of SO_2 on the properties of Schiff reagents has been considered by Ely and Ross (1949). They state that

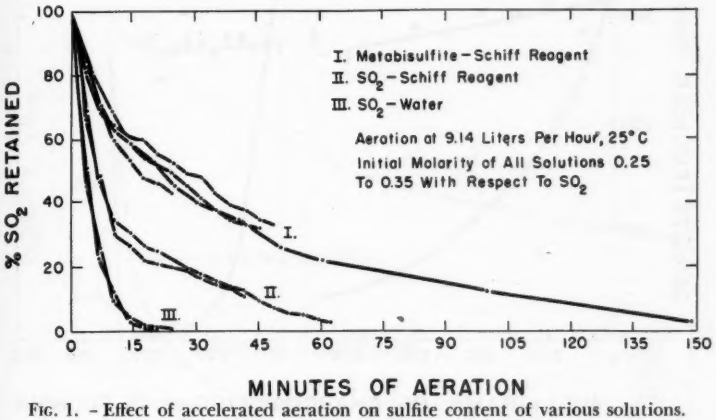

FIG. 1. – Effect of accelerated aeration on sulfite content of various solutions.

either N_2 or O_2, bubbled through a mixture of a Schiff reagent and partially hydrolyzed desoxyribonucleic acid, will prevent the development of color. With Schiff-formaldehyde mixture no such effect was obtained, so determination of the actual rate of SO_2 removal by this method was undertaken.

The SO_2 which remained in (1) a metabisulfite-Schiff reagent, (2) an SO_2-Schiff reagent, and (3) SO_2 water, after bubbling air through each for periods up to 1½ hours was measured by iodine titration. The rate of removal in all cases is greatly influenced by temperature. Typical findings under the same conditions in the three cases are shown in Fig. 1. Effectiveness of the SO_2-Schiff reagent with formaldehyde began to decline after 40 minutes aeration, but no decline was noted with the metabisulfite Schiff after 1½ hours.

High Concentration. At fuchsin concentrations of 0.5 or 1.0 g. per 100 ml. of reagent even saturation with sulfite did not produce any significant reduction of sensitivity with tissue sections. Ely and Ross have reported data indicating that the intensity of coloration produced in Schiff reagent by partially hydrolyzed desoxyribonucleic acid at least doubles each time the molar concentration of the sulfite in the reagent is doubled. Repetition of their experiment substituting formaldehyde as the colorizing agent showed by comparison no significant difference in intensity at sulfite levels other than the very low.

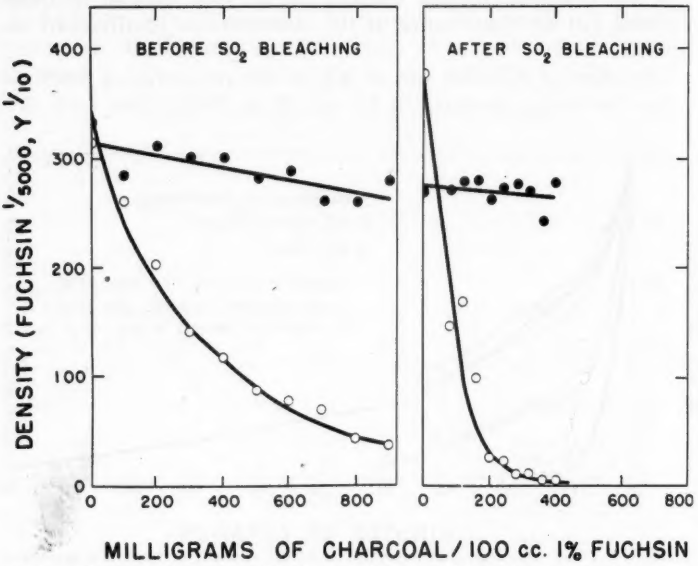

FIG. 2. — Charcoal adsorption of basic fuchsin (●) and its yellow contaminant (O).

While it may thus be seen that sulfite acting in conjunction with the leucofuchsin does not reduce the histochemical efficiency of Schiff reagents, pretreatment of aldehyde groups with sulfite, as often occurs with the Hotchkiss (1948) acid thiosulfate rinse, has been found to have distastrous effects on the staining produced in the Feulgen and periodic-Schiff technics, especially the latter.

2. FUCHSIN CONCENTRATION

The leucofuchsin content of a given Schiff reagent is subject to attrition from at least two sources, first from the loss of crystalline leucofuchsin which occurs during storage of the reagent and which may eventually account for a large part of the dye present, and sec-

ond from the removal of dye by charcoal in the final decolorization. In addition, large variations exist in the amounts of fuchsin specified in different Schiff reagent formulae. Two questions therefore arise: (1) What effect have differences in fuchsin concentration on the staining properties of Schiff reagents? (2) Can losses of dye, particularly by precipitation, be avoided within the range of concentration in which effective staining is obtained? The smaller loss occurring by decolorization is taken up in a separate section.

Relation to Staining Properties. Serial 2:1 dilutions with 0.15 *N* HCl of a Schiff reagent (prepared from 2 g. basic fuchsin, 3.8 g. $Na_2S_2O_5$, and 0.15 *N* HCl to make 100 ml.) showed no loss of staining potency at fuchsin levels as low as 0.125%, but further dilution

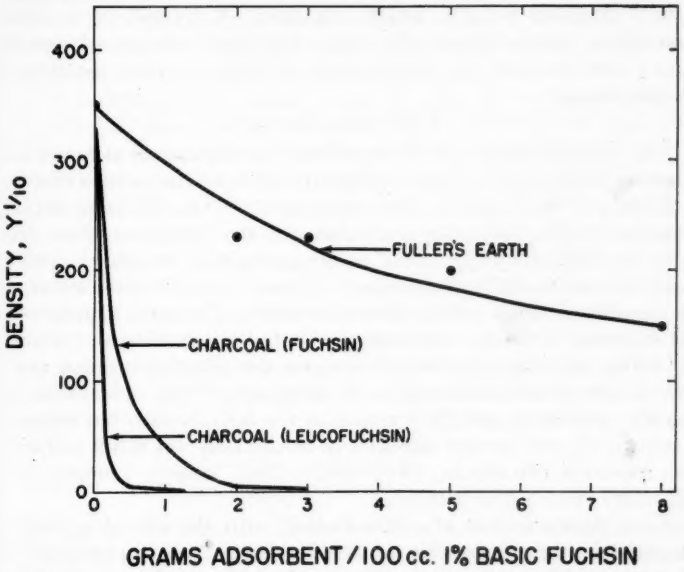

FIG. 3. — Comparison of removal of yellow contaminant (Y) from leucofuchsin solutions with Fuller's earth, and from leucofuchsin and fuchsin solutions with activated charcoal.

to 0.0625% showed a marked reduction of the ability to stain weakly periodic-Schiff positive materials such as collagen. Dilution with distilled water resulted in solutions staining at dye contents as low as 0.0078%, but, due probably to the rise in pH, the higher dilutions were pink and the possibility of nonspecific effects could not be excluded. It may be concluded that reliable Schiff staining is most likely to be obtained from reagents containing more than 125 mg. of basic fuchsin per 100 ml.

Conditions Governing Precipitation. Since detailed reports by other investigators concerning precipitation in Schiff reagents were not available, the following experiment was arranged to determine whether or not this effect resulted from our practice of dissolving the fuchsin in cold water or from the concentration of fuchsin employed. Five Schiff reagents were prepared by dissolving 1 g. or 0.5 g. of fuchsin in 100 ml. of hot or cold 0.15 *N* HCl, and for the fifth sample filtering the hot 1% solution, as is often specified. After bleaching to a clear yellow color with 1.9 g. $Na_2S_2O_5$, the residual color was removed by shaking with 300 mg. activated charcoal. After storage for one month in the refrigerator, only the 0.5 g. "cold" reagent was without precipitate, and after three months this reagent is still virtually without precipitate. Since this level of concentration is well above the point at which exhaustion effects appear, it seems that higher concentrations offer little advantage. This experiment of course does not rule out the existence of other variables significant in this respect.

3. DECOLORIZATION

The state of affairs which establishes decolorization of Schiff reagent as a *sine qua non* for its histochemical use has been stated by Scanlan and Melin (1937). They reported that "One of the principal obstacles to the successful application of the Feulgen technic has been the difficulty experienced in obtaining basic fuchsin. . . which could be satisfactorily decolorized. . . . Most of the trouble seems to be caused by a water soluble brown or yellow substance, of unknown constitution, which is not decolorized by sulfur dioxide and which, by diffuse staining, prevents or obscures the selective staining reaction of the fuchsin sulfurous acid compound." This observation is readily confirmed, and the removal of the contaminant has become a matter of even greater importance as the range of Schiff technics has increased (McManus, 1946, Lillie, 1947; Mowry, Longley and Millican, 1952; Lillie, 1952).

Final decolorization of sulfite-fuchsin with the aid of activated charcoal in the preparation of Schiff's reagent was introduced by Coleman (1938) and has since been virtually standard procedure. More recently Barger and DeLamater (1948) have stated that comparable results can be obtained by extracting the aqueous fuchsin solution with charcoal before treating with sulfite. No estimates of the practicality of the latter method have been published.

The observation that translucent solutions of fuchsin can be freed of the dye itself by treatment with activated charcoal raised some question as to the merits of the Barger-DeLamater method, and for want of information to the contrary, also raised the possibility that significant quantities of leuco-fuchsin are removed by Coleman's procedure.

Graduated amounts of charcoal equivalent to 0–900 mg./100 ml.

were added to 1% basic fuchsin and shaken for 2 minutes. By estimating spectrophotometrically (at 540 mμ) the amount of fuchsin remaining in each sample, then bleaching with SO_2 and determining the corresponding value for the yellow contaminant (at 464 mμ) the effectiveness of the Barger and DeLamater procedure was observed. For comparison 1% fuchsin solution was treated with corresponding amounts of charcoal *after* SO_2 bleaching. The relative amounts of yellow contaminant remaining were determined with the spectrophotometer directly, and the residual fuchsin was estimated in terms of the coloration produced (at 572 mμ) in 10 ml. of the bleached, charcoal treated solution in 1 hour by 1 ml. of strong formalin.

The results of this experiment are shown graphically in Fig. 2, which demonstrates clearly that charcoal has a considerably greater affinity for fuchsin than for leucofuchsin and is therefore not so selective with respect to the yellow constituent of the commercial fuchsin in unbleached solutions as it is in bleached.

The substitution of fuller's earth for charcoal in decolorizing as employed by Scott (1945) was tested on SO_2-fuchsin solutions with the spectrophotometric results shown in Fig. 3. The affinity for the yellow substance of the fuller's earth available in this laboratory is so low that effective decolorization does not appear to be possible. The effect with regard to the leucofuchsin concentration of the test solution was not determined.

From these data it is clear that the method of choice for decolorizing is still charcoal following the bleaching of the fuchsin to the leuco state. Amounts of charcoal required must be determined for each batch obtained. Our practice is to obtain a fresh supply every year, whether the old one has been exhausted or not, since a decline in effectiveness with time has been noted. The use of 300 mg./100 ml. is adequate if this precaution is observed.

4. HYDROGEN ION CONCENTRATION

Barger and DeLamater (1948) give the pH of their thionyl chloride Schiff reagent as 1.24 as against 1.38 they found for reagents prepared by the "usual method". The other pH values shown in Table 1 were determined in this laboratory, those reagents being chosen which seemed likely to show the greatest differences. Considerable variation was encountered, and experience with the different reagents showed that that of Alexander *et al.* (1950), if not others, had deficiencies which arose to some degree from the unsuitable pH of the solution.

Experimentally, no decline in histochemical effectiveness was observed on acidification of Schiff reagents even to as low a pH as that of 10 *N* HCl, although some decline of sensitivity *in vitro* was noted. Alkalinization of concentrated Schiff reagents containing 2 g. of fuchsin per 100 ml. resulted in precipitation of leucofuchsin at pH

3.0, and in weaker solution at slightly higher values, until at 0.25 g./100 ml. no precipitate formed at any pH. Thus the acidification of the turbid reagent of Alexander *et al.* corrects that shortcoming at once. Alkali precipitated leucofuchsin was readily dissolved not only by reacidification of the solution, but by further alkalization as well. In histochemical procedures no decline in effectiveness was observed until the pH of the reagents was raised to approximate neutrality, while at higher levels the reaction was appropriately localized although greatly weakened.

Discussion

In conclusion, the Schiff reagents used in chemical laboratories (e.g. Scott, Wertheim, *New Gould Dictionary)* are in general too low in fuchsin content to give reliable results in histochemical procedures. Though the Schiff reagents prepared especially for histochemical work are better suited to this purpose, no one conforms in all respects to the formulations which might be suggested by this study. It has not been found, however, that high SO_2 concentration is responsible for such shortcomings as any of them may have.

Our observations do make clearer the behavior which may be expected from Schiff variants and point the way toward a reagent offering the greatest economy of materials combined with maximum staining potency. On the basis of these observations we suggest the following procedure for making such a Schiff reagent: Without heating, dissolve 0.5 g. of basic fuchsin in 100 ml. of 0.15 *N* HCl to which 0.5 g. of $K_2S_2O_5$ (or $Na_2S_2O_5$) has been added. When the solution is no longer reddish (2 or 3 hours), decolorize by shaking with 300 mg. of fresh activated charcoal for 2 minutes and filter through Whatman No. 2 paper or the equivalent. The reagent is ready for immediate use, and if stored under refrigeration may be preserved for months.

I wish to acknowledge my indebtedness to Dr. R. D. Lillie for constant stimulation during the progress of this work through our many lunch hour conversations, and to Miss A. Laskey, Mrs. H. Burtner, Mrs. M. Duval, Mr. E. Henson and especially to Miss J. Greco for generous and skillful practical assistance.

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FEULGEN HYDROLYSIS WITH PERCHLORIC ACID

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ABSTRACT.—**In tissue fixed with Carnoy's acetic alcohol (1:3), the hydrochloric acid hydrolysis performed as part of the Feulgen reaction is optimal for only a very short period of time. When 10% perchloric acid is used as the hydrolytic agent, the same color maximum is obtained, and the optimal hydrolysis time at 25°C. extends from 12 hours to 24 hours. During this time the intensity of color does not change. The events which take place during the period of suboptimal hydrolysis are the same as those which take place during the corresponding period of hydrochloric acid hydrolysis. Part of the decrease in ultraviolet extinction of nuclei during the first 12 hours is due to the splitting off of purine bases from the desoxyribose nucleic acid. This is consistent with the increase in the amount of Feulgen dye bound by nuclei during this period of time. Between 12 hours and 24 hours no ultraviolet absorbing material is lost from nuclei, which is consistent with the fact that during this time the Feulgen color produced remains at a maximum.**

The Feulgen reaction (Feulgen and Rossenbeck, 1924) has become one of the most widely used cytochemical reactions. In addition to providing a method of staining chromatin by which the cytologist may study its morphology, it has been adopted by many workers who are interested in visualizing and accurately localizing desoxyribose nucleic acid within the cell. The reaction involves two separate and distinct steps: (1) mild acid hydrolysis, and (2) a reaction between the end product of hydrolysis and the Schiff reagent. The first step accomplishes the removal of purine bases from desoxyribose nucleic acid, which results in the formation of an aldehyde group on the desoxypentose sugar. The second step is merely a standard aldehyde reaction.

Bauer (1932) and later Hillary (1939) showed conclusively that hydrolysis time in 1 *N* hydrochloric acid at 60°C. is related to the type of fixative used. More recently (1948), using photometric technics to measure the amount of dye bound by nuclei of cartilage cells (frog tadpoles) fixed in acetic alcohol, the author has shown that optimal hydrolysis may be obtained for only a very short period of time. After this type of fixation the staining intensity falls off quite rapidly if the hydrolysis is allowed to proceed beyond this point. This is supported by the findings of Ris and Mirsky (1949) who, however, find a somewhat broader peak of color intensity in acetic-alcohol-fixed calf liver nuclei which had been isolated by the citric

acid method. Therefore, when fixation of this type is used, hydrolysis of the section must be rigorously controlled, especially if the color produced is to be used as the basis of any sort of estimate of desoxyribose nucleic acid. Such would not be the case if optimal hydrolysis could be accomplished over wide time limits. In order to obtain this type of hydrolysis one must choose either a fixative which will allow such treatment or use a different reagent to perform the hydrolysis. This paper is concerned with the use of perchloric acid as a hydrolyzing agent.

For this study, rat pancreas was fixed in Carnoy's acetic alcohol (1:3) overnight, and 3μ sections from the same ribbon were then

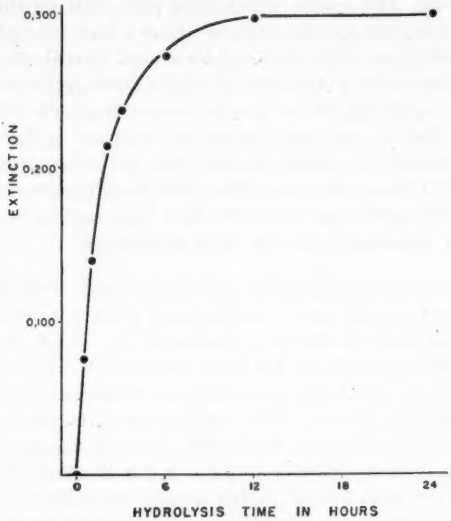

FIG. 1. — Absorption measurements of nuclei of rat pancreas cells, fixed in acetic alcohol, showing the relationship between duration of perchloric acid hydrolysis and the amount of color produced with the Schiff reaction. Each point represents a mean extinction of 10 to 20 nuclei.

subjected to treatment with 10% perchloric acid at 25°C. for varying periods of time. These were then placed for one hour in Schiff reagent which was prepared according to the method described by Stowell (1945). A slide which received no perchloric acid treatment was also placed in the Schiff reagent, as well as one which had been hydrolyzed by means of the standard procedure (12 minutes hydrolysis in 1 *N* hydrochloric acid at 60°C. was found to be optimal). The former served as a control for the perchloric acid hydrolysis while the latter was used for comparison with the maximal color produced by perchloric acid hydrolysis. In each case the amount of Feulgen dye bound by the nuclei was measured photometrically using an

apparatus[1] essentially the same as the one described by Pollister and Moses (1949). The nuclei were selected for size and shape; only spherical nuclei having diameters of about 6μ were used for measurements.

Results of measurements of Feulgen dye intensity are represented in Fig. 1. It may be seen that during the first 12 hours of hydrolysis with perchloric acid more and more color is developed in the nuclei. This reaches a maximum value after 12 hours of treatment ($E = 0.295 \pm 0.007$) and is maintained unchanged at the end of 24

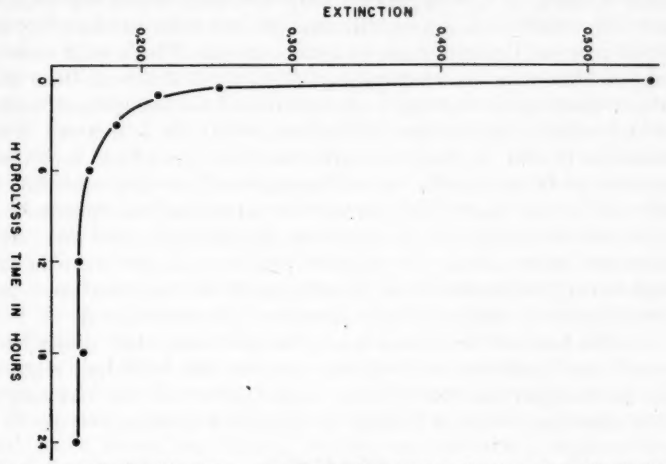

FIG. 2.—Relationship between nuclear ultraviolet extinction of acetic-alcohol-fixed rat pancreas and duration of perchloric acid hydrolysis. Each point is corrected for non-specific absorption as determined by means of a blank, and represents a mean extinction value of 10 to 25 nuclei.

hours hydrolysis ($E = 0.297 \pm 0.006$). This maximum color produced is not significantly different from that which is produced after the standard Feulgen procedure ($E = 0.301 \pm 0.007$). Since the transmission values of nuclei in the control section were found to be greater than 97%, which is not significantly different from 100%, correction of other measured extinction values was not deemed necessary.

In addition to measurements of Feulgen dye intensity, ultraviolet absorption measurements[2] were made of nuclei from sections adja-

[1] A Photovolt photometer (Mod. 512) with a standard B search unit was used for these measurements. A Farrand interference filter with a transmission peak at 560 mμ was used to isolate the desired spectral region from a Western Union zirconium arc lamp J 100.

[2] Measurements were made with a Photovolt electronic multiplier photometer (Mod. 512). The light source used was a Hanovia mercury lamp from which the 2537 A line was isolated by means of a Cooke double prism quartz monochromator.

cent to those used for Feulgen studies. These sections were mounted on quartz slides and were hydrolyzed in the same manner as were those used for measurement of regenerated fuchsin. A blank, for measuring the nonspecific absorption, was prepared by removing all nucleic acids with the hot trichloracetic acid extraction method described by Schneider (1945). Results of these measurements are expressed in Fig. 2. Here it may be noted, a decrease of ultraviolet absorption takes place during the first 12 hours of perchloric acid hydrolysis. The extinction of untreated nuclei was measured as 0.537 ± 0.011. At 12 hours of perchloric acid treatment it was reduced to 0.158 ± 0.005. It is during this time that the color produced by the Schiff reaction becomes more and more intense. This would seem to suggest that what is accomplished here is no different than that which takes place during a similar period of suboptimal hydrochloric acid hydrolysis (see Di Stefano, 1948; Ely and Ross, 1949). Between 12 and 24 hours of hydrolysis with perchloric acid there appears to be no further loss of ultraviolet absorbing material. At the end of this period the ultraviolet extinction was measured as 0.156 ± 0.005, which is not significantly different from the value measured after 12 hours hydrolysis. This is consistent with the fact that during this period there is no change in the maximal color produced by the modified Feulgen procedure presented herein.

On the basis of these data it may be concluded that, under these conditions, perchloric acid may be used for the hydrolysis required by the Feulgen reaction. Further experiments will be necessary to determine the effects of fixation on perchloric acid hydrolysis.

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SELECTIVE SILVER IMPREGNATION OF DEGENERATING AXONS IN THE CENTRAL NERVOUS SYSTEM

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ABSTRACT.—**Rat and rabbit brains containing surgical lesions of 5–10 days' duration were fixed in 10% formalin (neutralized with calcium carbonate) for 1 week to 6 months. Frozen sections (15–20 μ) were rinsed and then soaked 7 minutes in a 1.7% solution of strong ammonia in distilled water. Subsequent treatment was as follows: rinse; 0.05% aqueous potassium permanganate 5–15 minutes; 0.5% aqueous potassium metabisulfite, 2 changes of 2.5 minutes each; wash thoroughly in 3 changes distilled water; 1.5% aqueous silver nitrate, 0.5–1.0 hr.; 1% citric acid, 5–10 sec.; 2 changes distilled water; 1% sodium thiosulfate, 30 sec.; 3 changes distilled water. Each section is then processed separately. Ammoniacal silver solution (450 mg. silver nitrate in 10 ml. distilled water; add 5 ml. ethanol; let cool to room temperature; add 1 ml. strong ammonia water and 0.9 ml. of 2.5% aqueous sodium hydroxide), 0.5–1.0 min. with gentle agitation. Reduction of about 1 minute is accomplished in: distilled water, 45 ml.; ethanol, 5 ml.; 10% formalin, 1.5 ml.; 1% citric acid, 1.5 ml. Rinsing; 1% sodium thiosulfate, 10 sec.; thorough washing followed by dehydration through graded alcohol and 3 changes of xylene or toluene complete the staining process. Normal nerve fibers are slightly stained to unstained, degenerating fibers, black. The treatment in potassium permanganate is critical since too little favors overstaining of normal fibers and too much abolishes staining of degenerating fibers.**

The demonstration of axon degeneration of silver impregnation has proved to be of great value in experimental neuroanatomical research. For certain purposes, however, an important drawback of this research method may lie in the fact that, unlike the much older Marchi technic, the experimental silver technics (Glees, 1946; Nauta and Gygax, 1951) are nonselective, i.e. they impregnate normal and degenerating axons with approximately equal intensity. Although the simultaneous demonstration of both fiber categories is often beneficial, experimental study, particularly that of widely distributing fiber systems, is often rendered extremely laborious by the resultant necessity of examining extensive brain areas under high power magnification. Under such conditions scattered signs of axon degen-

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eration are easily overlooked in brain parts where nerve fibers of vastly different caliber are numerous. Furthermore, although disintegrating terminal axon ramifications may be clearly distinguishable, it is often difficult to trace degenerating proximal fibers which run inside massive fiber bundles. For such reasons, it would seem to be of some value to have available a selective silver technic, which would afford a more convenient localization of degenerative axon reactions.

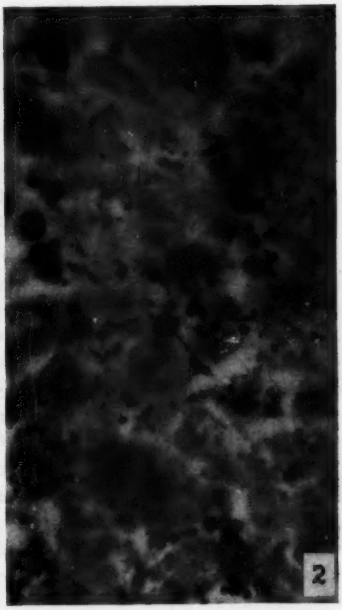
By the procedure described below, it has proved possible to obtain black impregnation of disintegrating axons in the rat's and rabbit's brain with normal axons stained various shades of brown (Fig. 1–3). Since the optimal fixation conditions appear to be the same for this method and the nonselective methods, the two procedures can be applied alternatively to the same material. By virtue of this fact, it has been possible to compare adjacent sections from experimental material stained by either of the two methods respectively. As the areas in which axon degeneration could be detected were the same for both technics, it seems likely that the results of the present method, besides being much more readily interpretable, are equivalent to those of the nonselective silver technics.

Procedure

1. Fix for 1 week to 6 months in 10% formalin, neutralized by calcium carbonate.
2. Cut frozen sections of 15 to 20 μ thickness. Sections can be stored in 10% neutral formalin for weeks.
3. Wash briefly and transfer sections for 7 minutes into dilute ammonia: 0.25 ml. of strong ammonia to 15 ml. of distilled water.
4. Wash briefly and bring sections for 5 to 15 minutes into a 0.05% solution of potassium permanganate, in which they obtain a brown color.
5. Decolorize sections in two consecutive volumes of a 0.5% solution of potassium metabisulfite, 5 minutes.
6. Wash very thoroughly in at least two dishes of distilled water, then:
7. Transfer sections for ½ to 1 hour into a 1.5% solution of silver nitrate.
8. Pass sections through a 1% solution of citric acid, 5 to 10 seconds.
9. Wash in three dishes of distilled water.
10. Transfer sections into a 1% solution of sodium thiosulfate, 30 seconds.
11. Wash in at least three dishes of distilled water.

Through steps 12 to 14 each section should be processed singly.

12. Bring section for ½ to 1 minute into an ammoniacal silver solution, to be prepared as follows:



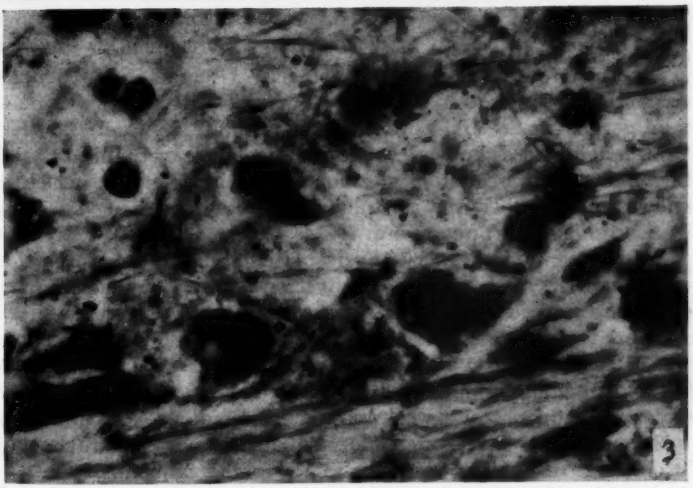


FIG. 1. — Degenerating axons in the capsula interna, 8 days after lesion of the cerebral cortex. × 500. Adult male rat.

FIG. 2. — Profuse terminal degeneration in the medial nucleus of the habenula. Degeneration time 8 days. × 900. Adult male rat.

FIG. 3. — Terminal degeneration in the lateral pontine nucleus, 10 days after lesion of the cerebral cortex. × 900. Adult female rat.

450 mg. of silver nitrate are dissolved in
10 ml. of distilled water.
5 ml. of pure ethanol are added. The mixture is allowed to cool to room temperature, and
1 ml. of strong ammonia water, and
0.9 ml. of a 2.5% solution of sodium hydroxide are added.

Throughout their stay in this solution, the sections should be moved about gently with a glass rod.

13. Without washing, transfer section quickly onto a solution composed of:

distilled water	45 ml.
pure ethanol	5 ml.
10% formalin[2]	1.5 ml.
1% citric acid	1.5 ml.

For even impregnation, it is essential that the section be allowed to float out rapidly on the surface of this reducing fluid. It acquires a brown color within 1 minute.

14. Wash briefly, pass sections through a 1% sodium thiosulfate solution (approximately 10 seconds) and wash again in three volumes of distilled water.
15. Dehydrate in graded alcohols. Prior to mounting, alcohol must be removed completely by using at least three consecutive volumes of xylene or toluene.

Discussion

The procedure described above essentially is a modified Bielschowsky method applied to frozen sections previously treated with potassium permanganate and potassium metabisulfite.

Although little is known concerning the actual mechanism of the Bielschowsky impregnation, its analogy with a similar principle known in photochemistry as "physical development" suggests that its two fundamental phases are:

I. Formation in the nerve fibers of silver nuclei, i.e. submicroscopic particles of either metallic silver or a silver compound. These nuclei are formed during the treatment of the section with silver nitrate.

II. Deposition around the nuclei of particles of nascent silver, released from an extraneous source (the ammoniacal silver solution) under the influence of a weak reducer like acidified formalin.

Normally, the silver nuclei formed during phase I are largely insoluble in thiosulfate solutions. Therefore, treatment of the sections with thiosulfate between phases I and II has no profound effect on the result of the procedure. However, in sections which have been treated previously with potassium permanganate and

[2]Commercial, non-neutralized formalin taken as 100%.

potassium metabisulfite as indicated in the description of the procedure, normal axons are no longer capable of forming insoluble silver nuclei during phase I, whereas disintegrating axons have retained this capacity. This is an inference from the experience that only disintegrating axons stain black when such sections are subjected to the procedure: phase I – thiosulfate – phase II.

The same difference between normal and degenerating axons was noticed in formalin-fixed sections, demyelinated with ammoniated alcohol, and subjected to phase I and thiosulfate treatment in dark red light. In such sections also, the final phase II fails to blacken the normal axons, while disintegrating axons are stained black. This observation suggests that bright light is one of the factors operating in the production of thiosulfate-insoluble silver nuclei by normal axons during phase I, and that disintegrating axons can form such or similar nuclei in the absence of bright light. Regulation of selective staining by means of the action of light proved to be less practical and less reliable than the technic given, hence the study of the phenomenon has been abandoned for the present.

Degeneration time. Significantly, optimal results were obtained only after relatively long survival periods: 5 to 10 days. It was found that for positive results the axon degeneration must have proceeded to the point of drop-like disintegration. Parts of the degenerating axons which have still retained their continuity fail to be impregnated. Mildly positive reactions were observed in the terminal ramifications of cut axons as early as two days after operation, while thicker fibers could not be traced until several days later.

Permanganate treatment. This step is one of the crucial parts of the procedure, and it seems subject to various hitherto undefinable factors. For instance, during periods of hot weather results were often entirely negative in this laboratory unless the length of the permanganate treatment was reduced considerably, sometimes even to 30 seconds with a 0.025% solution. Consequently, rather than to rely upon the prescription given, it is advisable to ascertain in a few preliminary experiments, the optimal duration of the treatment for each specimen separately. Generally, too short treatment with permanganate favors co-impregnation of normal fibers, while too intensive treatment prevents impregnation of fibers of either category.

Demyelination. Since best results were obtained in material which had not been in contact with alcohol-containing media, it is recommended to omit demyelination of the sections.

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LABORATORY HINTS
FROM THE LITERATURE

A DEPARTMENT DEVOTED TO ABSTRACTS OF BOOKS AND PAPERS FROM OTHER JOURNALS DEALING WITH STAINS AND MICROSCOPIC TECHNIC IN GENERAL

MICROTECHNIC IN GENERAL

SCOTT, T. L. **Wanted — an ideal mounting medium.** The Microscope, **8,** 206–9. 1951.

Solid mounting media available at the present time do not conform to the specifications of an ideal mounting medium. An ideal mounting medium should have the following characteristics: (1) good and rapid penetration of all objects; (2) neutral to stains and non-oxidizing; (3) tolerance of reagents used in preparing objects; (4) drying rapidly and evenly; (5) good adherence to glass; (6) should not fluoresce when observed with ultra-violet light; (7) should not produce small cracks nor crystallize on drying; (8) should have a suitable refractive index.—*G. Crossmon.*

DYES AND THEIR BIOLOGICAL USES

GETTLER, A. O., and SUNSHINE, I. **Colorimetric determination of alkaloids in tissues by means of methyl orange.** Anal. Chem., **23,** 779–81. 1951.

The main steps in the method, used chiefly on human tissues, are as follows: (1) extraction of alkaloids from tissue with boiling acidified water; (2) extraction of these alkaloids from the filtered aqueous solution by means of chloroform; and (3) formation of a chloroform-soluble colored compound of the alkaloids with methyl orange. The recoveries of most of the alkaloids and some of the antihistamines were good, although poor results were obtained with morphine.—*R. T. Whittenberger.*

HANSON, ROBERTA M., and GOULD, C. W. **Talc as an adsorbent for sulfonated azo dyestuffs.** Anal. Chem., **23,** 670–3. 1951.

In the usual procedure, dyes (such as Congo red and Congo corinth) were dissolved in water, adsorbed on a column of talc-Super-Cel, and caused to migrate by development with dilute solutions of pyridine in water. Excellent separations were observed. It is suggested that the process may involve competition between pyridine and cationic centers in the talc for the dye anions.—*R. T. Whittenberger.*

URONE, P. F., and BOGGS, W. E. **Acid-bleached fuchsin in determination of sulfur dioxide in the atmosphere.** Anal. Chem., **23,** 1517–9. 1951.

The method is simple, reliable, and determines sulfur dioxide in air in the presence of sulfur trioxide, hydrogen sulfide, and other impurities. The reagents used are: *Stock solution I:* To 228 ml. of water are added 22 ml. of concentrated sulfuric acid and 8 ml. of a 3% solution of basic fuchsin in ethyl alcohol. The solution is shaken for 30 minutes and 142 ml. of water is added. The solution is aged for 3 days, then filtered. *Stock solution II:* 5 ml. of 40% formaldehyde are diluted to 100 ml. with water. *Indicator solution:* One vol. of solution II is added to 10 vol. of solution I immediately before use.

Procedure: 10 ml. of a sampling solution (5% glycerol in 0.1 *N* NaOH) are placed in a bubbler and 10 liters of air are passed through it. One to 3 ml. of this solution (readjusted to 10 ml.) are mixed with 4 to 2 ml. of the indicator solution. This solution is held at 25°C. for a 30 minute color development period. The extinction of the color solution is measured on a spectrophotometer against

water at a wave length of 580 mμ. The method is standardized against a sodium bisulfite solution containing approx. 100 γ of sulfur dioxide per ml.—*R. T. Whittenberger.*

WEINBERG, JOSEPH; GREANEY, E. M.; RAWDINGS, B.; and HALEY, THOMAS J. **The use and toxicity of pontamine sky blue.** Science, **114,** 41–2. 1951.

The use of pontamine sky blue for delineating lymphatics is described, together with data indicating that the dye is classed as slightly toxic material. In intrathoracic surgery in man, 5 ml. of a 4% solution (0.2 g.) of the dye is injected into the lung parenchyma near the hilum. The dye spreads rapidly, outlining the lymphatics in the immediate as well as the more remote areas. The lethal dose for mice is 2.26 g/kg, hence there is a large safety factor when used in the above concentration in man.—*T. M. McMillion.*

YOUNG, H. Y., and GILL, R. F. **Determination of magnesium in plant tissue with thiazole yellow.** Anal. Chem., **23,** 751–4.

The procedure utilizes the color reaction of magnesium with thiazole yellow under alkaline conditions. The color is stabilized with polyvinyl alcohol and interferences are eliminated or compensated for by the addition of suitable agents. Results show increased sensitivity and reproducibility over those of other similar methods. Under favorable conditions, 10 samples may be analyzed in 20 minutes.—*R. T. Whittenberger.*

MICROÖRGANISMS

CASSEL, W. A. **Apparent fusion of the chromatinic bodies in species of Bacillus.** J. Bact., **62,** 514–5. 1951.

The author suggests that the apparent fusion of chromatinic bodies observed in young cultures of several species of *Clostridium* when exposed to air might be due to the accumulation of H_2O_2 in these organisms which lack catalase. By treating agar blocks on which cultures of *Bacillus cereus, B. pumilus,* and *B. mesentericus* were growing with various concentrations of H_2O_2 before fixation with OsO_4 vapor, hydrolysis with *N* HCl at 60 C. for 10 min., and staining 10 sec. with 0.1% basic fuchsin, apparent fusion was secured in the majority of the young cells. Untreated controls showed the usual pattern. Attempts to prevent fusion in two anaerobic strains of *Clostridium perfringens* by adding catalase were unsuccessful, but it seemed probable that the large molecules were unable to pass the bacterial membranes.—*Virgene Kavanagh.*

FISCHER, ROLAND, and SEIDENBERG, S. **Homologous mechanisms of bactericidal action and Gram-staining.** Science, **114,** 265–6. 1951.

This is a summary of the work of the authors and others in perfecting a technic for predicting the bactericidal effect against Gram-positive bacteria of water soluble, thermostable compounds by the determination of their affinity for wool. The determination is carried through by treating 1 g. of wool with 50 ml. of a neutral aqueous solution of 0.2 g. of the compound in question at 90°C. for 10 min. and weighing the wool sample to 10^{-4} g. Methods of degrading wool for the procedure and an explanation of the Gram-staining reaction are included.—*T. M. McMillion.*

LEIFSON, E. **Staining, shape, and arrangement of bacterial flagella.** J. Bact., **62,** 377–89. 1951.

A fairly simple method of demonstrating flagella has been developed to aid in the use of this character in bacterial classification. Since the flagella are submicroscopic, the stain is designed to be precipitated around them to bring them

into microscopically visible range. The method is as follows: Select bacteria from cultures grown under conditions suitable for the production of flagella (suboptimal temperature for growth, broth cultures for longer flagella and fewer detached ones); suspend bacteria in distilled water; wash by centrifugation to remove extraneous material and resuspend in distilled water; store in refrigerator for several days if desired – pathogenic forms in 2–4% formaldehyde; clean slides by soaking in saturated dichromate – sulfuric acid cleaning solution for a week; rinse thoroughly in tap and then distilled water; drain dry; store in dustproof containers. Prepare slide for smear by flaming for a few seconds in colorless flame of bunsen burner, side to be smeared next the flame; cool; make a heavy wax line across middle and along margin of distal half; place a medium large loopful of bacterial suspension at the end of the ringed half of the slide; tilt to cause liquid to flow to center waxed line; dry at room temperature; do not fix; add 1 ml. stain to smeared half of slide keeping it within the waxed confines; let stand for about 10 min.; rinse by placing directly under the faucet without pouring off the stain; counterstain with 1% aqueous methylene blue or with diluted Loeffler's methylene blue stain if desired; rinse; dry. The stain is prepared as follows: Mix equal parts of (1) 1.5% NaCl in distilled water, (2) 3% tannic acid in distilled water, and (3) 0.9% pararosanilin acetate and 0.3% pararosanilin hydrochloride in 95% ethanol or 1.2% basic fuchsin certified for flagella in 95% ethanol. The mixed stain is stable for a few days at room temperature and for several weeks in the refrigerator. The mixture of pararosanilins is indicated to secure the proper pH, though the acetate alone can be used. The quantity of stain added to the slide may be varied rather than the staining time. The apparent breadth of the flagella is controlled by a balance of the electrolyte concentration, the pH, and the relative solubilities of the tannic-acid-pararosanilin dye complex in alcohol and water.—*Virgene Kavanagh.*

MUDD, S., WINTERSCHEID, L. C., DELAMETER, E. D., and HENDERSON, H. J. **Evidence suggesting that the granules of mycobacteria are mitochondria.** J. Bact., **62,** 459–75. 1951.

The various methods of testing for specific enzyme systems associated with mitochondria in other organisms and the classical mitochondrial staining procedures have been applied to cultures of *Mycobacterium thamnopheos* and 4 strains of *M. tuberculosis* var. *hominis.* The methods were adapted and applied as follows: Preparation of smears:— Grow microcolonies on collodoin films on appropriate nutrient agars; remove a block of agar with the inoculation area and collodion with a flamed scalpel; fix in the vapor of 2% OsO_4 for 1 min. *(M. thamnopheos)* or 2 min. (virulent human strains); place clean cover slip over inoculation area and invert; flip off agar with scalpel; airdry film and inoculum on cover; dip scalpel in water and use it to peel off collodion, leaving inoculum intact; blot dry; mount in clarite or process as indicated in specific methods.

Flavoprotein dehydrogenase diaphorase by reduction of tetrazolium and neotetrazolium: Prepare a 1% aqueous solution of 2,3,5–triphenyltetrazolium chloride (TTC) or neotratrazolium chloride; sterilize by filtration or 15-min. autoclaving at 15 lb.; store up to 4 mo. in the dark; incorporate this stock solution in nutrient agar at a concentration of 0.005%; incubate *M. thamnopheos* at room temperature, *M. tuberculosis* at 37 C., preferably anaerobically; prepare slides as above at frequent intervals; after neotetrazolium, counterstain with carbol-fuchsin (Ziehl-Neelsen) diluted 1:2 with distilled water at room temperature 2 min. *(M. thamnopheos),* 8–10 min. *(M. tuberculosis).* To observe the reduction in living cells, place a small drop of cells in sterile distilled water on a thin film of neotetrazolium agar on a microslide, seal under a cover slip with melted paraffin, observe continuously. The reduced color with tetrazolium is red; with neotetrazolium, dark bluish purple. On agar plates tetrazolium appeared red in 2 hr. in

M. thamnopheos, 72 hr. in *M. tuberculosis.* In agar plates neotetrazolium appeared purple in less than 2 hr. with *M. thamnopheos,* 23 hr. with *M. tuberculosis;* in wet mounts the corresponding times were 5 and 15 min.

Cytochrome oxidase activity by Nadi reaction:— Prepare Nadi reagent (equal parts 1% aqueous solution dimethyl-p-phenylene diamine and 1% solution α-naphthol in 95% ethanol); add 5 drops each solution to 1 ml. cell suspension; prepare wet mounts at intervals on slide with cover slip sealed in place with melted paraffin; prepare permanent mounts by placing a drop of cell suspension on a block of hard agar, then follow general procedure. Indophenol blue is formed in areas containing cytochrome oxidase.

Mitochondria by Janus green B (#CJ–5, Coleman & Bell):— Grow cultures on agar containing Janus green B 0.05% or Janus green B and tetrazolium each 0.005%; prepare wet and dry mounts. Or add 0.0005% to 0.01% Janus green B to fairly heavy bacterial suspensions in distilled water. Mitochondria stain dark green and may later become red and finally colorless. In preparations from agar cultures, only the green color appeared. As cells settled from suspensions, rings of green, red, and white were observed.

Phospholipids with acid-hematin:— Prepare impression smears on cover slips coated with Mayer's albumin fixative; follow Baker's procedure (Quart. J. Micr. Sci., **87,** 441–70, 1946, and **88,** 463–5, 1947). Hematoxylin (National cert. # NH–7) was used for acid-hematin stain. Phospholipids are blue-black unless extracted with pyridine.

Mitochondria by Harman's stain (Stain Techn., **25,** 69–72, 1950): Prepare impression smears on Mayer's albumin fixative smeared cover slips; place in 4% solution fast green FCF(#Ngf–4) in 10% aniline water at 63°C. and allow to cool; remove after 8 min.; rinse in distilled water; place in 1% saturated aqueous solution picric acid, 10 min.; rinse; place in 1% aqueous solution phosphomolybdic acid, 2 min.; rinse; dehydrate and clear by passing at 2 min. intervals through 70%, 90%, and absolute ethanol, equal parts ethanol and xylene, and xylene to mount in clarite. The mitochondrial color is bluish-green.—*Virgene Kavanagh.*

HISTOCHEMISTRY

GLICK, DAVID; ENGSTROM, ARNE, and MALMSTROM, B. G. **A critical evaluation of quantitative histo- and cytochemical microscopic techniques.** Science, **114,** 253–8. 1951.

The utility and limitations of general procedures in quantitative histo- and cytochemical microscopic technics are discussed. The technics reviewed comprise some of the most serviceable and most elegant yet devised.—*T. M. McMillion.*

HOFFMAN, H. **The cytochemistry of bacterial nuclear structures. An examination of the Feulgen nucleal, Piekarski-Robinow, and ribonuclease technics.** J. Bact., **62,** 561–70. 1951.

The intracellular bodies of *Clostridium septicum, Fusobacterium polymorphum,* and *Lactobacillus* sp., as shown by basic dyes following hydrolysis with *N* HCl (Piekarski-Robinow) or with ribonuclease (Worthington Biochemical Laboratory, Freehold, N. J.), were larger than the granules within them that gave the Feulgen nucleal reaction or stained with methyl green in the methyl green-pyronin reaction (Stein & Gerarde, 1950, Science, **111,** 256–7). The author considers that a cortex of ribonucleic acid, not removed by acid hydrolysis or ribonuclease surrounds a central core of desoxyribonucleic acid. — *Virgene Kavanagh.*